A palmitoyl conjugate of insect pentapeptide Yamamarin arrests cell proliferation and respiration

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A B S T R A C T

A palmitoyl conjugate of an insect pentapeptide that occurs in diapausing insects causes a reversible cell-cycle arrest and suppresses mitochondrial respiration. This peptide compound also causes growth arrest in murine leukemic cell line expressing human gene *Bcr/Abl* and a farnesoyl peptide induces embryonic diapause in *Bombyx mori*. These results demonstrate that the insect peptide compounds can lead to the understanding of a common pathway in developmental arrest in animals and may provide a new peptidominetic analog in the development of biopharmaceuticals and pest management.

Keywords:

Insect pentapeptide

Yamamarin compound

Cell growth arrest

Mitochondrial respiration arrest

Biopharamaceutical

1. Introduction

Developmental or reproductive arrest (diapause) during seasons of environmental adversity is a widespread strategic event in many animals. Reduced metabolism and suppressed cell proliferation which facilitate survival under long-term environmental adversity, are important parts of the diapause syndrome. Developmental arrests have been examined in more than 100 mammals from different orders such as kangaroos,

rodents, mustelids and others. Developmental block occurs in many of them at an early stage of embryogenesis, around the time of blastocyst implantation. Although there is great diversity in the endocrine control of this process, growth factors that control the division and differentiation of blastocysts are conserved [20]. Microarray analysis revealed that the main functional categories of genes are differentially expressed in the dormant versus the activated blastocysts. Differences include genes involved in cell cycle regulation, cell signaling, and energy metabolic pathways [8].

Insects progress in their development through the embryonic, larval, pupal and/or adult stages and any block of this progression, the diapause, is regulated by endocrine interactions [3, 4]. The molecular controls of insect diapause were examined in the flesh fly by characterizing diapause-up-regulated and down-regulated genes [6]. The upregulation of the *hsps*, which was found in the diapause of several species, indicates linkage between diapause and expression of the stress proteins [21]. Diapause regulation is obviously different in the nematode *Caenorhabditis elegans* that responds to adverse conditions by halting development in the third instar long-lived dauer larvae. The decision to enter diapause may ultimately be mediated by *daf-9*, a cytochrome P450 of the CYP2 class related to steroidogenic hydroxylases and its cognate nuclear receptor *daf-12*, implying organism-wide coordination by lipophilic hormones [7].

These few examples demonstrate that specific diapause features and the organismic control of their manifestation are diverse. We do not know yet if there are similarities in the cell control of two basic features of arrested development, namely cell-cycle arrest and suppression of energy metabolism.

Using a palmitoyl conjugate of an insect pentapeptide called Yamamarin (DILRGa), we have previously shown that this compound causes a reversible arrest in the growth

of rat hepatoma cells [26]. The present results indicate that the compound functions as powerful and reversible arrestor of cell proliferation and respiration in the insect cells. Yamamarin provides valuable tool for elucidating molecular mechanism of the diapause arrest and a model for the development of new biologically active peptides.

2. **Materials and methods**

2.1. Preparation and modification of Yamamarin (GenBank accession no. P84863)

Peptides were synthesized on a PSSM8 peptide synthesizer (Shimadzu Co.), according to the FMOC solid phase strategy recommended by the manufacturer. Crude DILRGa obtained by the synthesis was purified by RP-HPLC on a Develosil ODS HG-5 column (Nomura Chemical Co., 10 mm × 250 mm, gradient elution from 0.1% TFA to acetonitrile containing 0.1% TFA, over 120 min, 1.0 ml/min). C16-Yamamarin (C16-DILRGa) (Fig. 1A) was prepared by the solid phase modification method. The DILRGa resin synthesized on the peptide synthesizer was treated with palmitoyl chloride in the mixture of DMF and pyridine. After cleavage, the reaction mixture was evaporated to dryness. The residue was extracted with 2-PrOH, after washing with hexane, a mixture of hexane and diethylether and ethylacetate, to obtain crude C16-DILRGa, which was purified by RP-HPLC.

Our previous paper [26] showed that DILRGa and ILRGa exert similar activities. Then farnesoyl-ILRGa (Fig.1B) was synthesized according to the liquid phase strategy. ILRGa was prepared on the peptide synthesizer, and the peptide freed from the peptide resin was used without purification. Ethyl farnesoate was prepared by the method of

Kulkarni et al. [15]. Although the product was a mixture of *trans, trans*- and *cis, trans*-isomers, it was used without separation. The ethyl farnesoate was hydrolyzed by treating with 1N NaOH. The hydrolysate was extracted with ethyl acetate after neutralization with 1N HCl. The extract was evaporated to give crude farnesic acid that was subjected to the coupling reaction with the peptide. The crude peptide and farnesic acid were mixed with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, and triethylamine in DMF. After stirring for 6 h at room temperature, the reaction mixture was evaporated to dryness. The residue was purified by RP-HPLC under the same condition to that used in the purification of C16-Yamamarin.

The purity of each synthetic product was proved by RP-HPLC analysis, and each product showed the appropriate m/z value as $[M + H]^+$ ion calculated for each product in MALDI-TOF-MS analysis on Kompact Discovery (Shimadzu Co.).

2.2. Cell proliferation assays

In accordance with a previous paper [1], *Drosophila* Schneider S2 cells were grown in Schneider's insect medium (Sigma) containing 10% FBS and supplemented with 1ml/L Antibiotic-Antimyeotic (100 ×) Liquid (Gibco). The cultures were incubated at 27°C to allow adhesion and growth, and seeded once per 4 days at an appropriate density. The cells were suspended in the culture medium at a density of 5×10^4 cells/ml. Aliquots of 200 µl of the suspension were dispensed into each well of a 96-well microtiter plate and pre-incubated at 27°C for 24 h. The medium was supplemented with the tested C16-Yamamarin dissolved in 0.5% DMSO. The control cultures were adjusted by the addition of the same concentration of palmitic acid. At desired time, 10

μl of the metabolic indicator WST-1 was added into culture medium [12], and the plate was kept at 27°C for 4 h. Cell density was accessed with a microplate reader as absorbance at A_{450} : the absorbance was directly proportional to the number of viable cells. Another assay of suppression of cell proliferation was used to count living cells (non-blue stained) in a hemocytometer under a light microscope and microscopic appearance of their cells was observed.

We also used cells obtained from the pupal ovaries of *Antheraea yamamai* [11] (*A. yamamai* cells). *A. yamamai* cells were cultured in MGM-448 (Modified Grace Medium-448) medium containing 10% FBS, and exposed to the same treatments as the *Drosophila* Schneider S2 cells. The cultures of rat hepatoma cells (dRLh84) were maintained at 37°C in humidified atmosphere containing 5% CO_2 according to Yang et al. [26, 27]. Exponentially growing cells were trypsinized, seeded at an appropriate density, and incubated for at least 24 h to allow adhesion and growth. Their microscopic appearance was recorded.

BaF3-*Bcr/Abl* cells were generated as reported previously [22]. BaF3-*Bcr/Abl* cells (2.5×10^5/200 μl/well) were grown in 10% FBS-supplemented RPMI 1640 medium in 96-well flat-bottomed plates and exposed to combination of C16-Yamamarin dissolved in 0.5% DMSO in the same medium at 37°C for 48 h. Cell proliferation assay was performed by the method, described before [2]. Murine bone marrow progenitor assay was performed according to the manufacture's manual (STEMCELL Technologies, Vancouver, BC, Canada). C16-Yamamarin was also added to methylcellulse medium (Methocult ® GFM3434, STEMCELL Technologies), supplemented with several hematopoietic growth factors (murine interleukin 3, human interleukin 6, murine steam cell factor and human erythropoietin) and used to culture murine bone marrow cells for

12 days with 5% CO_2 and 20% O_2. The number of colonies formed was counted using inverted microscopy.

2.3. Cell-cycle measurements

Drosophila Schneider S2 floating cells were suspended into the cell culture flask (75 cm^2) at a density of 5×10^4 cells/ml. After an incubation of 48 and 96 h in the presence of palmitic acid and C16-Yamamarin, the medium was removed and the cells were collected, counted, and fixed with ice-cold 70% ethanol, and then suspended in PBS containing 100 μg/ml RNAase A (Sigma). After the incubation at 37°C for 1 h, 100 μg/ml propidium iodide was added and the cells were analyzed by a FACS using flow cytometer equipped with a single 488 nm argon laser (Becton-Dickinson).

2.4. Digitonin-permeabilized cells and oxygen consumption assay

Digitonin-permeabilized *Drosophila* Schneider S2 cells were prepared by the method of Hofhaus et al. [10]. After 1 min exposure to 60 mM digitonin, the cell suspension was centrifuged at 1,000*g* for 5 min and resuspended in PBS at a density of 5×10^6 cells/ml. Oxygen consumption of permeabilized cells (5×10^7) was measured by the modified method of Piva and McEvoy-Bowe [19], using a Clark-type electrode cuvette (Oxy1, Hansatech Instrument Inc) at 27°C in a incubation medium. Incubation medium in air-saturated distilled water consisted of 130 mM KCl, 2 mM KH_2PO_4, 2 mM $MgCl_2$, and MOPS-NaOH (pH7.2). While the cell suspension and 5 mM succinate were added into the chamber, 6.25 to 100 μM C16-Yamamarin dissolved in DMSO (0 to 2 % of

final concentration) were introduced after adequate time for accurate slope measurement. Palmitic acid dissolved in DMSO and 1 mM KCN were used as a control and an inhibitor of cytochrome c oxidase respectively.

2.5. In vivo bioassay of embryonic diapause of the silkworm

A bivoltine race (Daizo) of the silkmoth, *Bombyx mori*, was used and female pupae destined to lay non-diapause eggs were prepared according to our previous paper [26]. Their subesophageal ganglia (SG) were extracted within 10 h after the pupal ecdysis to remove the source of diapause hormone [25], which is synthesized in SG. At 96 h after pupal ecdysis, farnesoyl-ILRGa dissolved in 5% ethanol (80 nmols/10 µl/pupa) was injected with a fine glass needle under the ventral side of females. Farnesoic acid was used as a control. Eclosed females were mated with untreated males and the oviposited eggs were scored in respect to the presence (in the dark coloration, embryogenesis is arrested at late gastrula stage) or absence (in the yellow coloration, non-diapause eggs to hatch within 10 days) of diapause. The proportion of diapause eggs was assessed by the counts of non-hatched eggs after 12 days of incubation.

3. Results

3.1. Insect and mammalian cells responding to C16-Yamamarin

In the present study we confirmed induction of growth arrest in the rat hepatoma cells, insect cells established from *Antheraea yamamai* ovaries [11] (*A. yamamai* cells),

and *Drosophila* Schneider S2 cells. Consist with our previous paper [26], we show that all cells exposed to the C16-Yamamarin exhibit reduced proliferation rate in comparison with cells grown in the control medium supplied with palmitic acid (Fig. 2A). Since Yamamarin was isolated as developmental arrestor from diapausing pharate first instar larvae of the Japanese oak silkmoth *A. yamamai* [23, 27], we have now examined effect of Yamamarin on the cells from this source. The growth of isolated cells is very slow and 23.6% of reduction of their proliferation was detected with 25 μM C16-Yamamarin, only after a 7-days incubation (Fig.2B and Supplementary Fig. S1A). We also tested growth arrest in *Drosophila* Schneider S2 cells to unravel which genes are significantly upregulated and downregulated by this peptide. When these cells were incubated in medium with 12.5 μM C16-Yamamarin for 48 h, cell proliferation was reduced by 17.6% (Fig. 2C and Supplementary Fig. S1B). These results indicate that both mammalian and insect cells are very sensitive to C16-Yamamarin.

In another experiment we examined reversibility of the growth arrest in *Drosophila* Schneider S2 cells. After a 48 h incubation in the presence of 12.5 μM C16-Yamamarin, the medium was removed and replaced with a fresh control medium. During subsequent 24 h incubation, the cells recovered to 73.8% of the original mitotic activity. Interestingly, the cells treated with the C16-Yamamarin in the 3rd-consecutive culture again decreased their proliferation to 51.9% level and in the 4th-consecutive culture in the control medium they recovered to 84.5% (Fig. 2D). Observation of the cells under a phase contrast microscope also confirmed a temporal arrest of their proliferation after each exposure to C16-Yamamrin (Supplementary Fig. S2A). From these results we concluded that C16-Yamamarin has a capacity of reversible suppression of cell proliferation in insect cells, as shown in the mammalian cells [26, 27].

3.2. Gene expression profile exposed to C16-Yamamarin

We next examined whether reversible arrest and growth in *Drosophila* Schneider S2 cells (Supplementary Fig. S2A) are associated with different patterns of gene expression. We performed whole-genome microarray analysis using AFFYMETRIX *Drosophila* genome 2.0 array and obtained a complete gene expression dataset of 18,952 entries.

The most affected genes are shown in Supplementary Fig. S2 in relation to functional categories and treatment. Analysis of 10 such genes reveals the major and functional categories responding to C16-Yamamarin : 1) minichromosome maintenance 7 (*Mcm7*), 2) latheo (*lat*), 3) DNA replication primer (*DNAprim*), 4) ecdysone-inducible gene L3 (*lmpL3*), 5) pyruvate dehydrogenase (*Pdk*), 6) bruno-2; translational regulation in *D. melanogaster* (*bru-2*), 7) *Drosophila* homolog of human members of the serine/arginine-rich (SR) protein family (*B52*), 8) ribosomal protein L28 (*RpL28*), 9) human double-stranded RNA-activated protein kinase-like ER kinase (*PEK*), and 10) suppressor of variegation 3-9 (*Su(var)3-9*). Comparing with differentially expressed genes between dormant and activated blastocysts in mice [8], there are a few genes of the same family such as *Mcm5* in mice and *Mcm7* in *Drosophila* cell cycle, and *Eno1* in mice and *Eno* in insect cells of carbohydrate metabolism (Supplementary Table S1). We did not detect highly coordinated cascades between mice and insect active and resting cells, but the down-regulation of functional categories associated with the cell cycle, DNA replication reactome, and energy metabolism are common to the developmental arrest events in many species. The results of microarray analysis encouraged us to investigate whether the cell cycle is decelerated by down-regulation of 43 cell-cycle-related genes, including *Mcm7* and *lat*.

3.3. Cell-cycle phase and oxygen consumption exposed to C16-Yamamarin

When Schneider S2 cells were exposed to C16-Yamamarin, an arrest of the cell cycle occurred at any detectable stage (Fig. 3B to D). In the case of the suppression of rat hepatoma cells (dRLh84), Yamamarin substantially and specifically increased the G0/G1 phase fraction [27]. It is worth mentioning that the brain cells of diapausing *Sarcophaga crassipalpis* are also arrested in G0/G1 stage and that the *pcna* gene is critical for shutting down the cell cycle [4]. The stage of the cell cycle arrest may be different in insect species, and diapausing mammalian blastocyst cells are arrested in G0 or G1 phase [20]. Cell cycle arrest is the major diapause syndrome in many animals, but its regulation at the gene level is unknown.

We also tested the respiratory capacity of digitonin-permeabilized *Drosophila* Schneider S2 cells, according to the method of cell treatment with a detergent (digitonin) which permeabilizes selectively the plasma membrane and leaves the mitochondria, other cell organelles, and the cytoskeleton substantially intact [10]. In the digitonin-permeabilized cells, the succinate-dependent respiration decreased in the presence of 100 μM C16-Yamamarin to less half from 18.7 nmol O_2 consumption/min/5 × 10^7 cells in the control (Fig. 3E). After the 1st measurement, the digitonin-permeabilized cells were collected by a light centrifugation, suspended in a fresh medium, and also for the consecutive measurement. Oxygen consumption of the consecutive control represented 47.0% of the 1st measurement, and the 2nd oxygen consumption of the digitonin-permeabilized cells exposed to C16-Yamamarin in the 1st measurement was not significantly in comparison to controls. In the 3rd measurement, KCN, an inhibitor of cytochrome c oxidase, was added to the media and oxygen

consumption was monitored. In contrast to C16-Yamamarin, KCN inhibited the succinate-dependent respiration completely (Fig. 3F). Hence, the suppressive effect of C16-Yamamarin on the mitochondrial respiration is reversible just as the effects on the cell proliferation. These results indicate that C16-Yamamarin induces an apparent and reversible reduction of the oxygen consumption in the digitonin-permeabilized cells and this function could reflect a diapause-like feature in insect cells.

3.4. Applied effects of Yamamarin compounds

Aside from a clue to understanding the molecular mechanism of animal diapause, C16-Yamamarin could provide two possible scenarios relevant for biopharmaceuticals and pest management. We investigated growth arrest in murine leukemic cell line (BaF3) expressing human *Bcr/Abl* (BaF3-*Bcr/Abl*), which is the responsible for human chronic myeloid leukemia. BaF3-*Bcr/Abl* can proliferate without any cytokines, while BaF3 proliferation requires murine interleukin 3. The signaling pathways in BaF3-*Bcr/Abl*, which mediate the autonomous growth, are considered to be similar as those in human chronic myeloid leukemia cells. Therefore we evaluated the biological effects of C16-Yamamarin on the proliferation of BaF3-*Bcr/Abl*. It suppressed the proliferation of BaF3-*Bcr/Abl* in a dose-dependent manner (Fig. 4A). We also evaluated the biological effects of C16-Yamamarin on primary culture of murine bone marrow cells. C16-Yamamarin exhibited suppressive effects on the proliferation of murine bone marrow hematopoietic progenitor cells in a dose-dependent manner (Supplementary Fig. S3). These results suggest that C16-Yamamarin inhibit several signaling pathways,

including activated *Bcr/Abl* induced pathways in human chronic myeloid leukemia cells and proliferation- and maturation-related pathways in normal hematopoiesis, probably by the similar mechanism as those in insect cells containing *Drosophila* Schneider S2 cells.

The embryonic diapause of *Bombyx mori* is induced by the diapause hormone (DH, 24 amino acids with an amidated C-terminus) secreted in pupal stage from a dozen of female neurosecretory cells [25]. We found that the injection of C16-Yamamarin into female pupae induces embryonic diapause in the silkmoth, *B. mori* [26]. Such a targeted control of diapause with an inexpensive and topically acting chemical would be of great practical potential for the control of harmful insects. Thus we synthesized a farnesoyl-ILRGa that holds cell growth suppressive activity by tetrapeptide truncated at the N-terminus [18, 27]. This compound was injected into female pupae of the non-diapause-egg producing *B. mori* that was used as a model of harmful lepidopterans. The treatment induced embryonic diapause (Fig. 4B). There is an apparent similarity between the action of C16-Yamamarin and farnesoyl-ILRGa, suggesting that the molecular mechanism of embryonic diapause is similar to the C16-Yamamarin effects on cultured cells. However, the mechanism of DH action and its possible interplay with C16-Yamamarin has not been elucidated.

4. Discussion

Diapausing organisms become hypometabolic as a consequence of metabolic depression. In embryonic diapause of the brine shrimp, *Artenia franciscana*, ADP levels

remain constant and possibly prevent caspase activation in the post-mitochondrial pathway, which may lead to metabolic arrest and the prevention of cell death [9]. Theses events might be a feed-back consequence of the molecular change occurring downstream in the cytoplasm. The mechanisms of signaling and subsequent depression of mitochondrial capacity have not been addressed. C16-Yamamarin can provide a clue to the solution of this problem (Fig. 3E). As described in Supplementary Table S1, all 27 TCA cycle-related genes were down-regulated and not even one up-regulated gene was detected.

The metabolic and the cell proliferation arrests throughout diapause are tightly correlated with a depression of mitochondrial respiration that causes an energy-limited state [9, 16]. Virtually nothing is known about the common arrestors of cell proliferation and mitochondrial respiration and associated block of apoptotic and necrotic cell death. We, however, found that C16-Yamamarin inhibits cell proliferation and mitochondrial respiration and brings cells to a state similar to diapause. The concentrations required for the inhibition of mitochondrial respiration were 8-fold higher than that needed for the suppression of cell proliferation (Figs. 2 and 3). Different permeabilitites of the plasma and mitochondrial membranes for C16-Yamamarin may be a possible explanation and C16-Yamamarin may function like a cell-permeable peptide across the plasma membrane [13]. In addition, the microarray analysis disclosed the remarkable up-regulation of ribosomal proteins (Supplementary Fig. S2B), indicating that C16-Yamamarin may also trigger unidentified pathways in the cytoplasm and nucleus. In spite of these uncertainties, C16-Yamamarin can be used as a tool to understand how diapausing organisms survive long periods of adverse conditions.

When Yamamarin was evaluated by the cardiotropic test on the semi-isolated heart of

another insect species *Tenebrio molitor*, it showed a strong cardioinhibitory effect [24]. Yamamarin compounds also induced embryonic diapause in the eggs laid (Fig. 4A) [26]. These findings will encourage us to challenge the research to develop new agrochemicals penetrating through the insect cuticle for the control of harmful insects [17]. Together, our results of grow arrest in murine leukemic cell line expressing human gene *Bcr/Abl* offer attractive potential for the development of pharmaceutical agents that may contribute to improvement of human health [5].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online.

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Figure Legends

Fig. 1. The structures of C16-Yamamarin and farnesoyl-ILRGa. C16-Yamamarin in our previous paper [26] and farnesoyl-ILRGa were synthesized as described in materials and methods.

Fig. 2. Phase-constant micrographs of insect and mammalian cells responding to the treatment with C16-Yamamarin by reversible arrest of cell proliferation. The cells were seeded into 100 mm dishes and cultured described as in Methods. (A) Rat hepatoma cells (dRLh84) were cultured in the presence of palmitic acid (control) or C16-Yamamarin. By comparison with the control cells (insert), the presence of 25 μM C16-Yamamarin reduced survival and proliferation, consistently with the results of our previous study [26]. Initial cell density was 2.5×10^4 cells/ml. (B) Cells obtained from pupal ovaries of *Antheraea yamamai* were suspended at density of 5×10^4 cells/ml. Since their growth was so very slow, the incubation time was expanded to 168 h. At that time, there was a clear difference between the poor performance of these cells in the presence 25 μM C16-Yamamarin in comparison with the controls (insert). (C) The growth of Schneider S2 cells was comparable to that to the rat hepatoma cells. Their proliferation in 48 h (insert) was clearly inhibited with 12.5 μM C16-Yamamarin (Supplementary Fig. S1 shows that the effect was dose-dependent within 3.125 to 25 μM concentrations). These results indicated that Schneider S2 cells are very sensitive to C16-Yamamarin. Scale bar = 200 μm. (D) Cell proliferation in Schneider S2 cells was assessed with the metabolic indicator WST-1 and the growth of cultures treated with 12.5 μM C16-Yamamarin was expressed in % of cell counts in the control culture that was treated with the same dose of palmitic acid dissolved in 0.5% DMSO (abscissa).

The assay culture (each bar) was exposed to C16-Yamamarin for 24 h (violet bar) to 48 h (red bar) (marked as + 1st culture) and then for 24 h (light blue bar) to the control medium (marked as - 2nd culture, total consecutive incubation of 72 h). In 24 h (pink bar) of the 3rd-consecutive culture (marked as + 3rd culture, total consecutive incubation of 96 h) the cells were treated again with C16-Yamamarin and afterwards they were grown in control medium for another 24 h (gray bar) (marked as – 4th culture, total consecutive incubation of 120 h). Results are means ± SE of 3 independent replicates. *p <0.05, **p< 0.001 versus each control.

Fig. 3. Cell-cycle phases and oxygen consumption in *Drosophila* Schneider S2 cells exposed to C16-Yamamarin. The cells were cultured in media containing 12.5 and 25 μM concentrations of palmitic acid and C16-Yamamarin, respectively. Cells were counted as used in methods and cell-cycle phases were determined by a FACS after 48 and 96 h. (A), number of cells (× 10^5/ml); (B), rate of G0/G1; (C), rate of S; (D), rate of G2/M. (E), Inhibitory effect of C16-Yamamarin on oxygen consumption in digitonin-permeabilized cells. (F), When 100 μM C16-Yammarin reduced oxygen consumption to half of the control value, the cells were collected and suspended in a fresh medium. Their oxygen consumption in the 2nd-consecutive culture was measured in the absence and presence of KCN. All data are means ± SE of 3 independent samples. *p < 0.005, **p< 0.001 versus each control.

Fig. 4. Yamamarin effected growth suppression in murine leukemic cell line expressing human *Bcl/Abl* gene and induction of embryonic diapause in the silkworm. (A), Murine leukemic cell line (BaF3) expressing *Bcl/Abl* (BaF3-*Bcl/Abl*), which is responsible for

human chronic myeloid leukemia. The rate of cell proliferation was assayed by the methods described as in materials and methods. A dose-dependent manner of C16-Yamamarin was found in the cells incubated for 24 h (violet), 48 h (red) and 72 h (light blue). Results are means ± SE of eight wells. (B), Non-diapause egg producers were prepared and females 96 h after pupal ecdysis were injected with 80 nmols of farnesoyl-ILRGa (right, F-ILRGa) and farnesoic acid (left, F) dissolved in 5% ethanol. Oviposited eggs were scored in respect to the presence (brown coloration) or absence (yellow coloration) of diapause. The injection of farnesoyl-ILRGa induced 16.5 ± 5.3% of diapause in the laid eggs of three independent experiments.

Figure

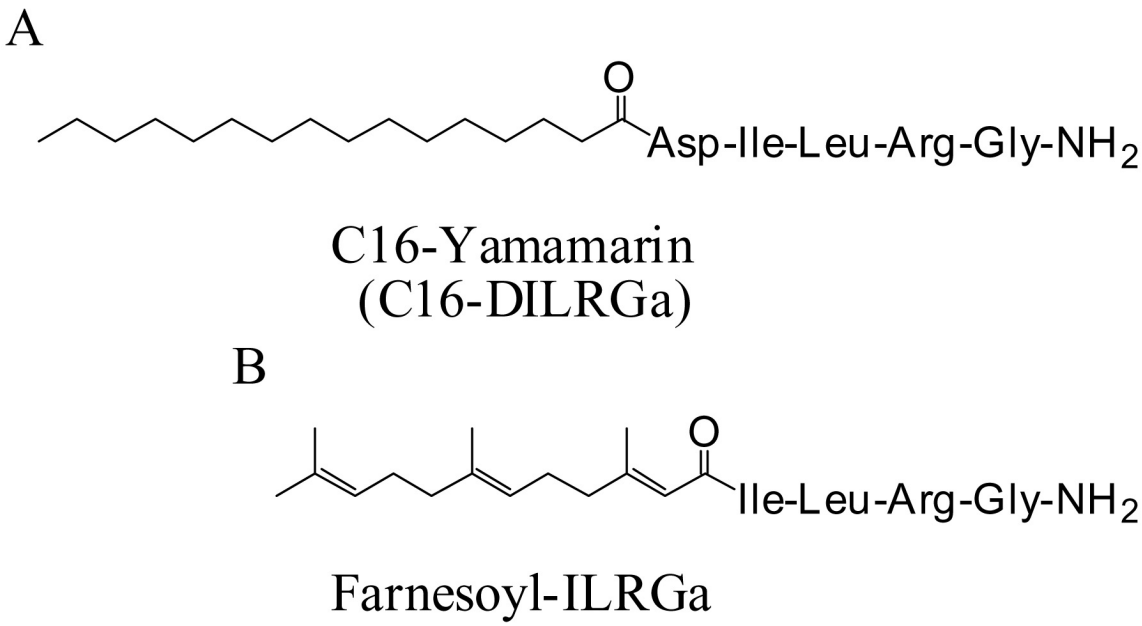


Fig. 1. Sato *et al.*

Figure

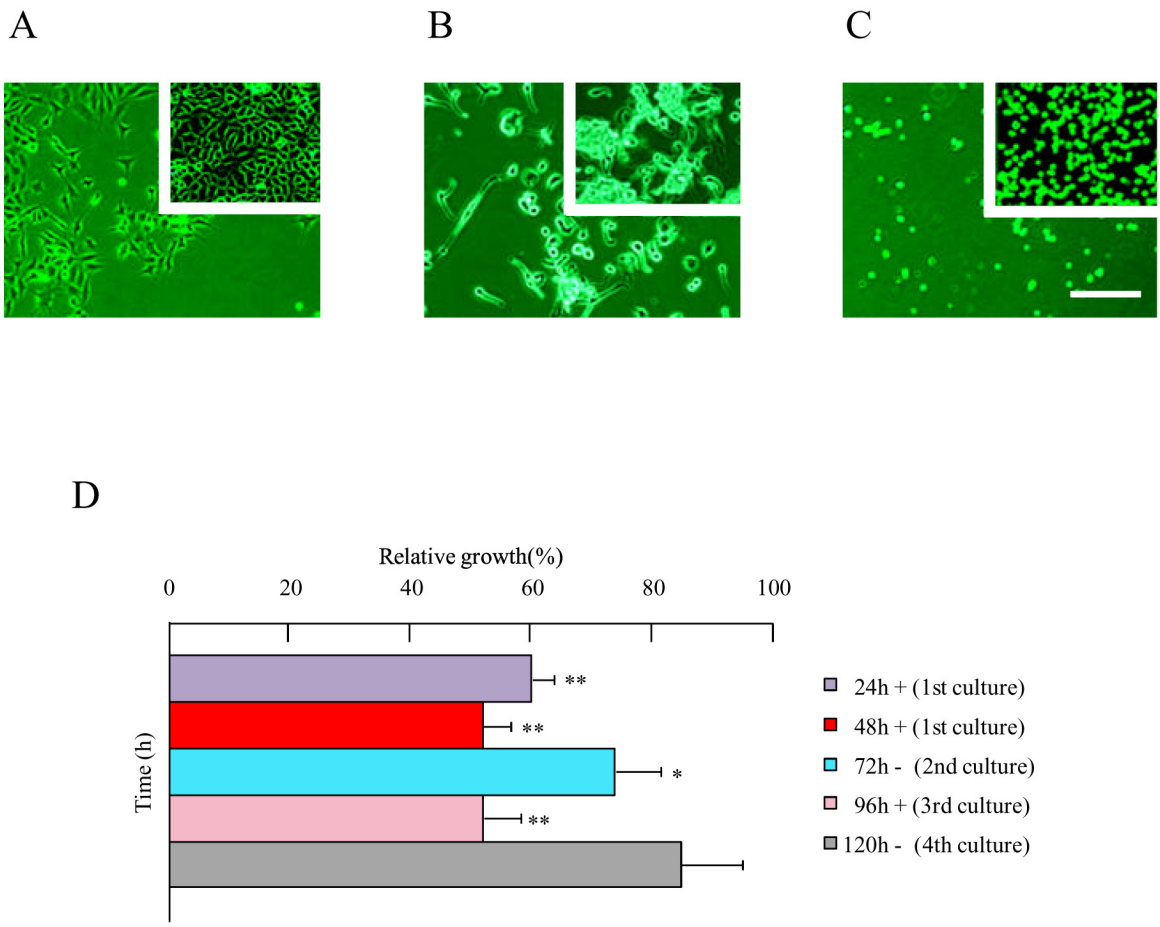


Fig. 2 Sato *et al.*

Figure

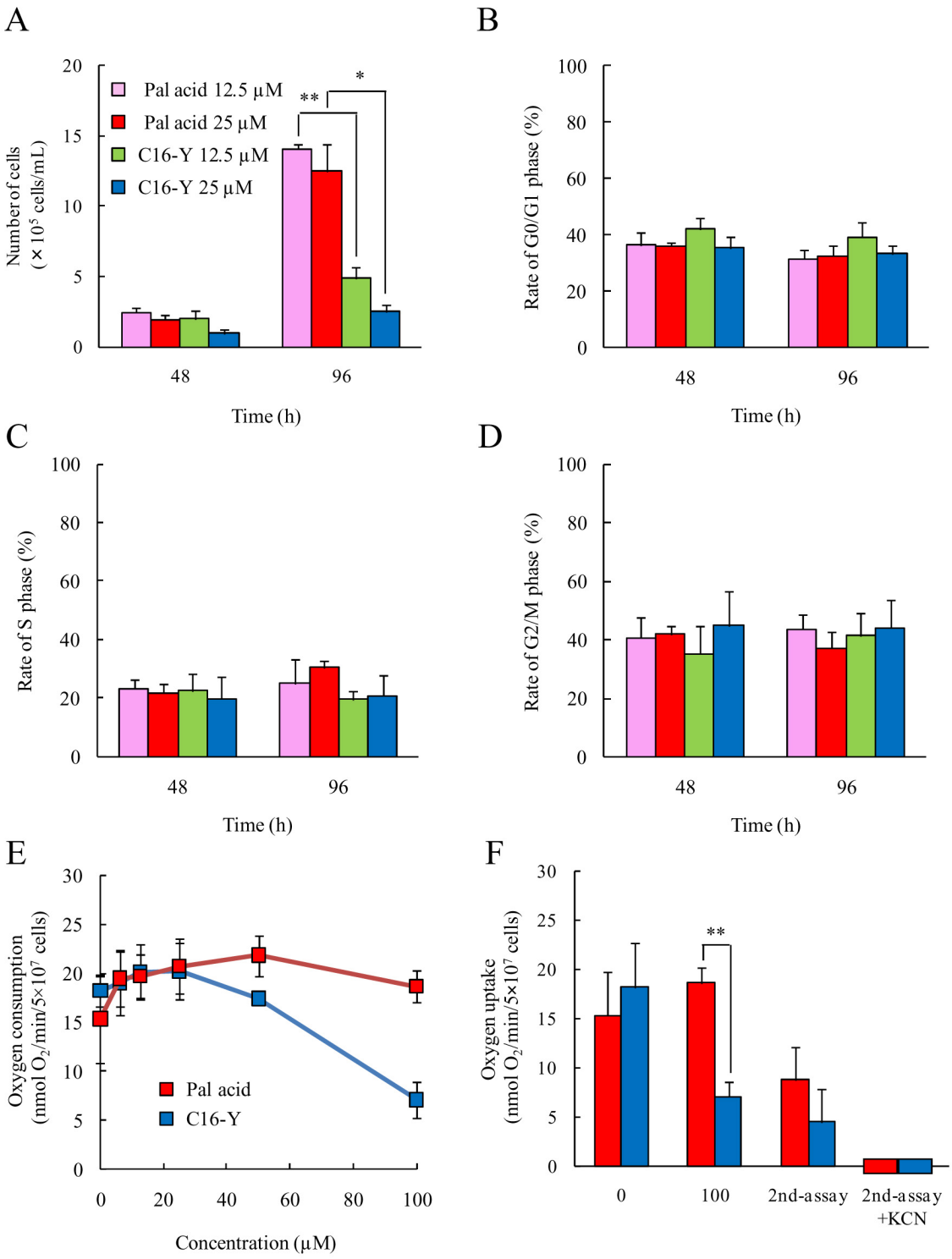
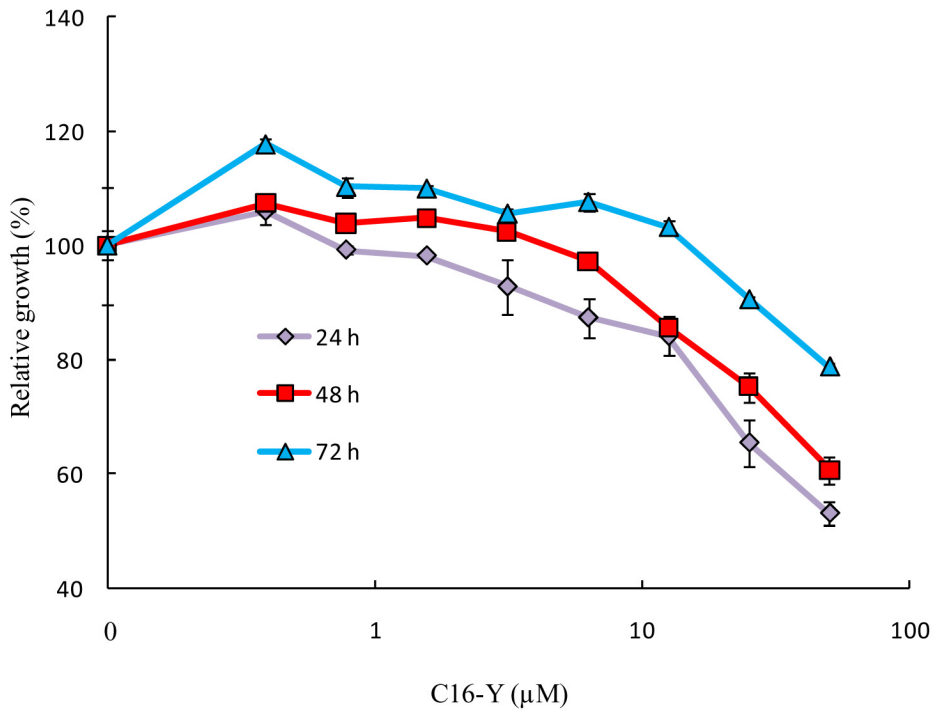


Fig. 3 Sato *et al.*

Figure

A



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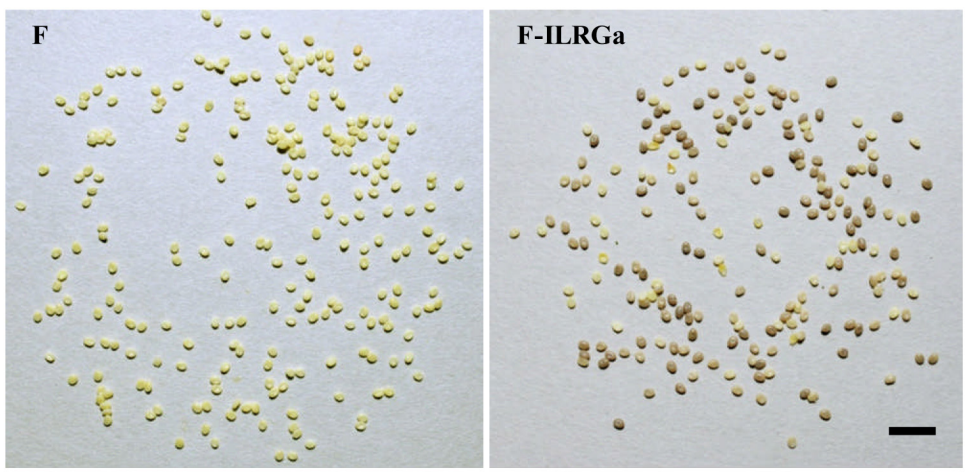

Fig. 4 Sato *et al.*