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Research Article

Monopolar Spindle Induced by Isoamericanol A Suppresses Human Breast Cancer Cell (MCF-7) Growth

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Abstract

Background and Objective: The neolignan isoamericanol A was purified from the organic layer of the MeOH extract of the waste residue of the *Jatropha curcas* L. (Jatropha) seed. The previous work revealed that isoamericanol A can induce G2/M arrest on MCF-7. The aim of the present study was to evaluate the selective effect of isoamericanol A on the human breast cancer cell line (MCF-7), as well as to clarify the point at which isoamericanol A exhibited anti-cancer activity on MCF-7 at M phase. **Materials and Methods:** The normal human breast cell line (MCF-10A) and the human breast cancer cell line (MCF-7) were subjected to isoamericanol A treatment for 3 days for cell growth inhibition assay. DNA microarray analysis was performed to reveal the expressional difference on MCF-7 with or without 3-day isoamericanol A treatment. Also, the effect of short term isoamericanol A treatment was studied by flow cytometry analysis, as well as immunofluorescent staining using Aurora B, α-tubulin and the chromosome. **Results:** In this experiment, both MCF-10A and MCF-7 were treated with isoamericanol A to measure the relative degrees of inhibition. Though inhibiting both cell lines, isoamericanol A effected 25.9±6.14% (n = 4, $p<0.05$) more growth inhibition on MCF-7 than it did on MCF-10A. Microarray analysis showed isoamericanol A resulted in numerous expressional changes in cell division related genes pertaining to spindle formation. The immunofluorescent staining showed that brief isoamericanol A treatment is capable of inducing cell growth inhibition specifically by disrupting regular spindle formation during M phase. Flow cytometry analysis showed brief isoamericanol A treatment induced G2/M arrest. **Conclusion:** Isoamericanol A inhibits human breast cancer cell proliferation more than it does normal human breast cells. Moreover, this study revealed that isoamericanol A treatment can induce G2/M cell cycle arrest due to monopolar spindle formation during cell division.

Key words: Isoamericanol A, neolignan, human breast cancer cell line (MCF-7), immunofluorescent, aurora B, spindle formation

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Oil rich seeds from Jatropha cultivated in the tropical and subtropical areas have gained popularity as a carbon dioxide-free fuel resource[1]. The process of biodiesel production from the seeds, however, leaves a large quantity of defatted byproduct residue[2]. Since almost all parts of Jatropha trees have long been known as traditional folk remedies for various sicknesses in African and Asian countries[3,4], It is practical to find medicinal usage for the waste remnant of the Jatropha seeds, samples of which were provided by Dr. Tanachai Pankasemsuk from Chiang Mai University, Thailand, a sister school with Kagawa University.

In search of use for the byproduct, the neolignan isoamericanol A (Fig. 1) was identified in the MeOH-organic constituent of the defatted seed residue, which possesses the highest antioxidant activity among 4 types of extracts[5,6]. Hence, it was considered to have the greatest promise in terms of discovering useful bioactivity. The previous study reported that synthetically prepared isoamericanol A inhibits cell proliferation in a dose-dependent manner on the human cancer cell lines of breast cancer (MCF-7 and MDA-MB231), hepatocellular carcinoma (HuH-7) and cervical cancer (HeLa)[7]. Distinguished inhibition was first observed on the last of 3 days of treatment[6,7]. Therefore, the molecular mechanisms behind prolonged 3-day 75 μM isoamericanol A treatment on MCF-7 were investigated with DNA-microarray analysis, flow cytometry, TUNEL assay, western blot and quantitative real-time PCR. The results indicated that isoamericanol A promotes G2/M cell cycle arrest, hindering further cancer cell growth[3]. Yet, the specific disruption point during G2/M phase had still not been identified and thus required a visual parameter for differentiating G2 from M.

This experiment was conducted to provide additional information regarding the anti-cancer properties of isoamericanol A on MCF-7. Though it was shown to exhibit anti-proliferative characteristics with MCF-7, the degree of growth inhibition had not been compared with the regular human breast cell line (MCF-10A). Additives were applied to MCF-10A and MCF-7 to evaluate the degrees of inhibition during their proliferations.

The flow cytometry used in the previous studies was insufficient in differentiating the G2 and M phases from one another[8,9]. Detailed analysis of the microarray results revealed that there were decreased expressional changes in numerous genes related to cell division (M phase of the cell cycle) by isoamericanol A treatment. Therefore, this study focused on the specific role of isoamericanol A during M phase. For the process of proper cytokinesis during M phase, correct mitotic chromosomal separation is ensured by proper kinetochore attachment to the microtubule polymers, leading to construction of a regular spindle[10]. The microarray results suggested isoamericanol A perturbs M phase progression on MCF-7. Aurora B kinase (AURKB) was listed as one of the cell division related genes in the microarray analysis. AURKB, known for changing its location of expression during cell division[11] was used as a phase marker to observe mitosis in MCF-7 for the immuno fluorescent-stain study. Current study explored the relationship among Aurora B, α-tubulin and the chromosome/DNA in regulating spindle formation.

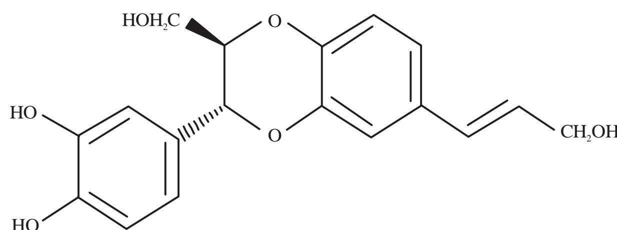

Fig. 1: Structure of isoamericanol A

MATERIALS AND METHODS

Experimental preparation for isoamericanol A: The experiment was conducted as a collaborative international research project between the Faculty of Agriculture and the Faculty of Medicine at Kagawa University from April 2012 to present. Isoamericanol A was synthesized[12] and prepared using the method as reported prior[7].

Cell culture: MCF-10A cells (JCRB Cell Bank, NIBIOHN (Osaka, Japan)) were cultured in accordance with the previously described methods[13]. All of the growth factors were purchased from Sigma-Aldrich. MCF-7 breast cancer cells (JCRB Cell Bank, NIBIOHN (Osaka, Japan)) were also routinely cultured as explained in the earlier works[6,7].

Anti-proliferative and cytotoxic activities: Anti-cancer ability was tested by modifying the method reported[6,7]. MCF-10A (2500 cells/well) and MCF-7 (4000 cells/well) were subjected to 0 and 300 μM isoamericanol A treatment for cell proliferation assay. The relative extent of inhibition was compared between the two types of cells after 3 days of isoamericanol A treatment. Cell proliferation rate was monitored by applying CCK-8 (Dojindo Molecular Technologies. Inc., Tokyo, Japan). Its reaction with in cells was measured with an ultraviolet-visible spectrophotometer at 450 nm. The changes for the 4 trials were calculated by contrast to the control.

DNA-microarray analysis: Following the Materials and Methods section of the previous work[7], MCF-7 cells treated for 3 days by either 0 or 75 μM isoamericanol A were subjected to microarray analysis.

Immunofluorescent microscopy: MCF-7 cells were harvested in an 8-well chamber slide at 37°C for observation of cell division. The following day, the cells were treated with thymidine for 16 h of synchronization. Then, after a period lasting between 5 and 8 h after 0 or 75 μM isoamericanol A treatment, the cells were fixed with 4% paraformaldehyde and permeabilized with a mixture of 10% goat serum and 0.1% Triton X-100. Cells were stained with a mixture of monoclonal mouse anti-α tubulin antibody (1:1000; Sigma-Aldrich) and monoclonal rabbit anti-AURKB antibody (1:250; Abcam). The reaction was followed by the mixture of goat anti-mouse IgG H and L, Alexa Fluor® 488, (1 400; Thermofisher Scientific) and Goat Anti-Rabbit IgG H and L, Alexa Fluor® 594, (1:200; Thermo fisher Scientific). The cells were also counterstained with To-PRO-3 (1:1000; Thermo fisher Scientific). Images were acquired with 63×Plan-Apochromat 1.4 oil differential interference contrast (DIC) objective (numerical aperture 1.4) in a Carl Zeiss LSM 700 (CLSM) microscope equipped with 488 and 555 nm lasers and were analyzed with the Zen 2009 software package (Zeiss, Germany).

Flow cytometric analysis: Applying the method described in the previous work[7], the cells were synchronized with thymidine for 16 h after a day of cell cultivation. MCF-7 samples were harvested for 8, 10 and 12 h treatment of 0 or 75 μM isoamericanol A.

Statistical analysis: Data were expressed as mean±standard error (SE) from 3-4 independent trials. The significance of difference between the control and sample-treated groups was evaluated by Student's t-test, using excel software (Microsoft). Statistical significance was set at $p<0.05$.

RESULTS

Degree of inhibition on MCF-10A and MCF-7: The effect of 300 μM isoamericanol A was evaluated on MCF-10 A and MCF-7 over 3 days (Fig. 2). With the proliferation rate for control as the standard, the isoamericanol A treatment inhibited cell proliferation by 17.2±3.43% (n = 4; $p<0.05$) on MCF-10A and 43.1±4.56% (n = 4; $p<0.05$) on MCF-7 (Fig. 2).

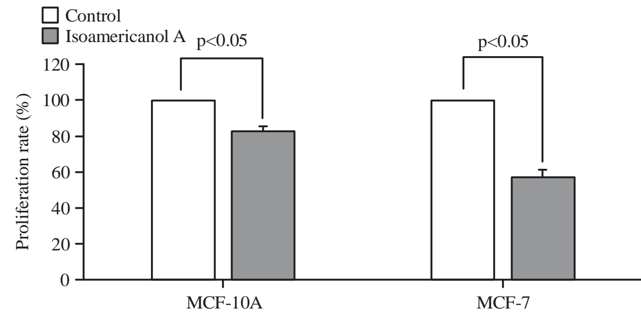

Fig. 2: Anti-cancer properties of isoamericanol A on MCF-10A and MCF-7

Table 1: Potential genes responsible for improper spindle formation by isoamericanol A treatment

Gene	Gene description	Expression change
KIF18A	Kinesin family member 18A	0.43
SKA1	Spindle and kinetochore-associated protein 1	0.43
MAD2L1	Mitotic spindle assembly checkpoint protein	0.43
SGOL1	Shugoshin-like 1 (S. Pombe)	0.47
AURKB	Aurora B kinase	0.50

M phase related genes from microarray analysis: There was a total of 188 MCF-7 genes which resulted in 2-fold more or half-fold less expression due to isoamericanol A treatment. Though the previous study showed only cell cycle related genes, microarray data analysis tool categorized 106 genes either cell cycle related or cell division related[7], though many of those of overlap with one another. Here, among cell division related genes observed in the results, Table 1 showed potential genes which were observed as possible candidates responsible for disrupting cell division.

Immunofluorescent staining of M phase cells: The short-term effect of isoamericanol A on MCF-7 was triple-stained by α-tubulin (green), AURKB (red) and DNA (blue) during the spindle formation in MCF-7 cell division (Fig. 3). Figure 3a showed normal cell division: AURKB located at the chromosomal arm during prophase (5 h after thymidine synchronization). Next, it integrated at the spindle equator at metaphase (6 h). Then, it accumulated in the spindle midzone at anaphase (7 h) and relocated at the midbody for the final process of cytokinesis (8 h). Figure 3b showed when isoamericanol A was applied: the majority of metaphase cells (at 6th h) formed a monopolar spindle where 2 opposing polar ends come close together. The cell division continued to anaphase (at 7th h), then to telophase (at 8th h).

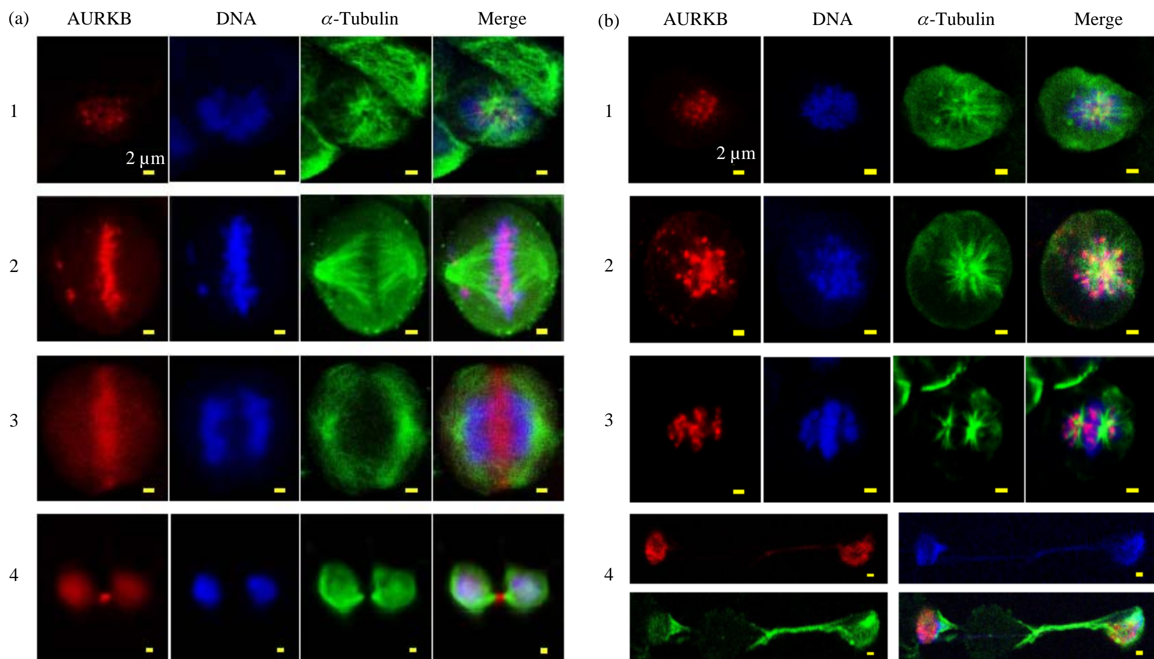

Fig. 3(a-b): Monopolar spindle formation of MCF-7 by isoamericanol A. The morphology of MCF-7 with (a) Non-treated and (b) Isoamericanol A were compared by stages of cell division: (1) Prophase, (2) Metaphase, (3) Anaphase and (4) Telophase

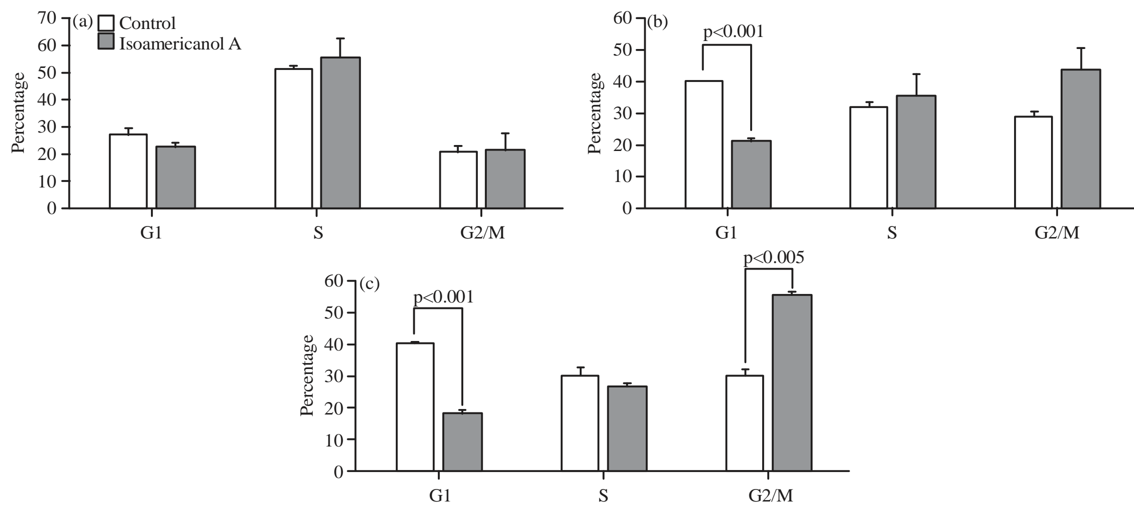

Fig. 4:(a-c): Flow cytometry analysis of brief isoamericanol A treatment on MCF-7. Isoamericanol A induced MCF-7 to arrest at the G2/M stage by the time of 12 h treatment

Flow cytometric analysis of brief isoamericanol A treatment on MCF-7: There was not a significant percentage difference across G1, S and G2/M with or without isoamericanol A treated Fig. 4a until the 10th h of treatment (Fig. 4b): the G2/M percentage was 28.7±1.67 (n = 3) in the control and 43.3±6.89 (n = 3) in the isoamericanol A treated group. At the 12th h of treatment (Fig. 4c), the G2/M percentage was 29.9±2.21 (n = 3) in the control and 55.1±1.47 (n = 3) in the isoamericanol A treated group.

DISCUSSION

In this study, the inhibitive properties of one of the extraction constituents, the neoligan isoamericanol A from the Jatropha seed was evaluated on the MCF-10A and MCF-7 cell lines. Isoamericanol A showed a moderate inhibitory effect on MCF-7 growth. However, it still had a 25.9±6.14% (n = 4, $p<0.05$) greater inhibitive effect on MCF-10A. In clinical treatment, etoposide, the semisynthetic derivative of the lignan podophyllotoxin is utilized against small cell lung carcinoma and testicular cancer[14]. Unfortunately, a certain level of cytotoxicity is observed to afflict normal cells[8]. It has been a challenge to find a way to ease side effects without diminishing the desirable cancer drug effect. This may be possible by introducing chemical modifications to isoamericanol A.

The genes strongly related to M phase were re-evaluated in this study (Table 1). Kif18A[15,16], SKA1[17,18], MAD2L1[19,20], SGO1 (aliases; SGOL1)[21], AURKB[11,22] are all known as important genes that are responsible for ensuring proper bipolar spindle formation. They are essential for stabilizing the kinetochore/microtubule interaction, thus allowing proper chromosome segregation to proceed[11,15-22]. While numerous possibilities were considered as to why isoamericanol A disrupted cell division, the decreased gene expression in these 5 genes implied that it interferes with proper chromosome positioning and/or sabotages proper chromosomal connection to the microtubules at the right time before the cells enter cytokinesis.

MCF-7 was immunofluorescent stained after a short time with or without isoamericanol A treatment. Normally, the bipolar mitotic apparatus provided structural basis for equal chromosome segregation, forming 2 new daughter cells (Fig. 3a). However, there is a case where the mitotic chromosomes are clustered at a single pole (monopolar oriented)[23]. When isoamericanol A was applied (Fig. 3b), the majority of metaphase cells (Fig. 3b-2) formed a monopolar spindle where 2 opposing polar ends come close together. Therefore, the chromatids are not placed at the equator plate at metaphase. The cell division continues, yet only the chromatids with a connection to α-tubulin are connected are carried on to anaphase (Fig. 3b-3). However, without proper expression of the genes which are important for cytokinesis, such as AURKB, the two daughter cells move towards opposite ends, dragging their connection (Fig. 3b-4). Isoamericanol A interrupted MCF-7 growth at M phase by inducing inaccurate spindle formation.

Flow cytometry analysis was performed because the previous flow cytometry analysis only showed G2/M arrest with long term isoamericanol A treatment (3 days)on MCF-7[7]. In this experiment, clear G2/M arrest was observed by brief 12th h isoamericanol A treatment on MCF-7. There was a time discrepancy between when monopolar spindle formation was induced during immuno fluorescent staining (5-8 h) and when isoamericanol induced G2/M arrest in flow cytometry (10-12 h). It is likely that this is simply due to variance in experimental preparation for each cell sample. Overall, the immunofluorescent staining revealed improper spindle formation responsible for hampering proliferation. This supports the flow cytometry results of G2/M arrest obtained in this experiment by brief isoamericanol A treatment on MCF-7.

CONCLUSION

This study introduced the functional mechanism of the neolignan compound isoamericanol A, identified in the defatted Jatropha seeds, on MCF-7. It was found to inhibit human breast cancer cell proliferation more than normal human breast cells. Moreover, this study revealed that the inhibition of breast cancer cell proliferation is triggered by isoamericanol A treatment, which can induce G2/M cell cycle arrest due to monopolar spindle formation during cell division.

SIGNIFICANCE STATEMENT

This study discovered an anti-cancer function of isoamericanol A that may lead to a beneficial for the usage for defatted Jatropha seeds. The oil rich seeds have great potential as a environmentally sustainable substitute fuel in the presence of global climate change. Finding a practical use for the seed waste promotes not only economic improvement, but also provides hope for effective new cancer treatment. This study will help to guide critical research for implementation of chemotherapy drugs from seed byproduct.

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