1 Javamide-I-O-methyl ester increases p53 acetylation and induces cell death
2 via activating caspase 3/7 in monocytic THP-1 cells
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5 **ABSTRACT**
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7 *Background:* Javamide-I and-II are phenolic amide compounds found in *Coffea sp.*

8 Previous study suggested that javamide-II may be a potent compound with Sirt1/2
9 inhibition activity.

10 *Purpose:* However, the effects of javamide-I and the its O-methyl ester on Sirt
11 inhibition, p53 acetylation and cell death have not been investigated.

12 *Methods:* The isolation and synthesis of javamide-I and its O-methyl ester analogue were
13 confirmed by NMR. Their potential effects on Sirt1/2/3, p53 acetylation and cell death
14 were examined using sirt assay, *silico* analysis, Western blot, caspase 3/7 and apoptotic
15 assay methods.

16 *Results:* Javamide-I and its O-methyl ester demonstrated a similar inhibition pattern;
17 Sirt1 (IC₅₀ of 19 µM) better than Sirt2 (IC₅₀ of 104 µM) and Sirt3 (IC₅₀ of 160 µM).

18 However, javamide-I and its O-methyl ester were found to inhibit Sirt1 better than Sirt2,
19 which is different from javamide-II able to inhibit Sirt2 stronger than Sirt1. In *silico*

20 analysis, javamide-I and its O-methyl ester showed a competitive binding pattern against

21 NAD⁺, which was also supported by the kinetic analysis with K_i = 20.1 µM for

22 javamide-I and 19.5 µM for the O-methyl ester. However, the O-methyl ester increased

23 p53 acetylation better than javamide-I in monocytic THP-1 cells. Since caspase 3/7

24 activation is often followed by the p53 activation, we investigated their effects on caspase

25 3/7, and found that O-methyl ester again activated caspase 3/7 greater than javamide-I in

26 the cells, eventually leading to apoptotic cell death.

1 *Conclusion:* These data suggest that the O-methyl modification in javamide-I may play a
2 critical role in increasing p53 acetylation, activating caspase, and eventually inducing the
3 THP-1 cell death.

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2 *Short tile:* Javamide-I and its O-methyl ester as Sirt inhibitors

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4 *Keywords:* Javamide-I-O-methyl ester, Coffee, Molecular docking, Sirtuin inhibition,
5 Caspase3/7, THP-1 cell death

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7 *Abbreviation:* Sirt, Sirtuin; HPLC, High Performance Liquid Chromatography; NMR,
8 Nuclear magnetic resonance; NAD^+ , Nicotinamide adenine dinucleotide; ANOVA,
9 Analysis of variance;

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1. Introduction

Worldwide, coffee is considered as one of most consumed beverages (Charrier et al, 1985). Recent studies indicated that coffee may have beneficial effects on several chronic diseases such as liver disease, inflammation, diabetes, cancer, and neurodegenerative diseases (Cano-Marquina et al, 2013; Ludwig et al, 2014; Cornelis, 2015; Butt and Sultan, 2011). In fact, coffee contains many compounds such as caffeine, chlorogenic acids, niacin, trigonelline, cafestol, and phenolic amides, potentially relevant to the purported effects (Casal et al, 2000; Alonso-Salces et al, 2009). In particular, javamide-II (*N*-caffeoyltryptophan) is a phenolic amide compound found in coffee, recently being demonstrated to inhibit Sirt1/2, (Park, 2016). Although coffee also contains javamide-I besides javamide-II, the potential effects of javamide-I and its derivative O-methyl ester on Sirt inhibition and p53 acetylation have not been examined. Furthermore, their potential effects on cell death have not been investigated, even though the activation of p53 often leads to apoptotic cell death. Therefore, in this paper, javamide-I was isolated from coffee and its analogue O-methyl ester was chemically synthesized. Then, their chemical structures were verified using NMR and their effects of on Sirt1/2/3 were first investigated using *in vitro* assay and *silico* analysis. Additionally, we investigated their potential cellular effects of javamide-I and its O-methyl ester on p53 acetylation in monocytic THP-1 cells, because the inhibition of Sirt1/2 frequently leads to increasing p53 acetylation (Reed, 2015). Finally, caspase 3/7 activation and apoptotic cell death were studied in the same cells, since the activation of p53 is regularly followed by caspase activation which can induce apoptotic cell death (Shen, 2001).

1 **Materials and methods**

2 **Materials**

3 Tryptophan, coumaric acid, dichloromethane, and N, N-dimethylformamide and other
4 chemicals, were purchased from Sigma Chemical Co. (St. Louis, MO).

6 **Methods**

7 **Isolation of javamide-I from coffee.**

8 Javamide-I was isolated from coffee samples using the equal mixture of four
9 different coffee brands, as described previously (Park, 2016). The isolated javamide-I
10 was further purified by HPLC (Waters, Milford, MA) and used for NMR analysis.

12 **Chemical synthesis of javamide-I-O-methyl ester**

13 The synthesis of javamide-I-O-methyl ester was performed using a method
14 previously described (Park, 2016, Park, 2012). Briefly, coumaric acid was dissolved in
15 dimethyl sulfoxide (DMSO) and converted to the symmetrical anhydride with 1,3-
16 diisopropylcarbodiimide (DIC). Tryptophan was esterified in methanol and modified with
17 benzyl chloride and added to the reaction mixture. The reaction mixture was incubated at
18 room temperatures with a gentle stirring for 12 h. The synthesized products were
19 recovered, treated with anhydrous HF, and purified by HPLC (Waters, Milford, MA), and
20 used for NMR analysis.

22 **NMR analysis**

23 For NMR experiments, the sample was prepared by dissolving javamide-I and

javamide-I-O-methyl ester (20 mg) in d6-DMSO (0.75 mL). ^1H and ^{13}C spectra were acquired at room temperature on the JEOL BCX-400 NMR spectrometer operating 400 MHz for ^1H and 100 MHz for ^{13}C . Chemical shifts were referenced to DMSO (2.50 ppm for ^1H , 39.5 ppm for ^{13}C).

Sirt1/2/3 assays.

The Sirt1, 2 and 3 inhibition assay was performed using sirt1/2/3 direct fluorescent screening assay kits (Cayman Chem., MA), according to the manufacturer's manual. Briefly, human sirt1/2/3 in the kit was incubated with javamide-I and javamide-I-O-methyl ester. Then, the fluorescent substrate and NAD^+ were added to the reaction mixture. The fluorescence emitted by the deacetylation of Sirt1/2/3 was analyzed at an excitation wavelength (350 nm) and an emission wavelength (450 nm).

***Silico* and kinetic analysis**

Silico analysis was performed using ICM-pro (MolSoft, San Diego, CA). The projected binding potency to the active pocket of the Sirt1 enzymes was measured as the best ranked scoring with the most favorable free binding energy ($\Delta G_{\text{binding}}$). Kinetic analysis of the Sirt1 inhibition was performed using Lineweaver-Burk plot. Data points in all figures represent the mean $\pm$ SD (n = 3). For kinetic study, this assay was performed by diluting NAD^+ to the indicated concentrations.

1 **Cell culture**

2 THP-1 cells were obtained from ATCC (Manassas, VA) and grown in RPMI
3 supplemented with 10% (v/v) heat-inactivated fetal bovine serum at 37 °C in a
4 humidified atmosphere of 5% CO₂.

6 **Assays for p53 acetylation.**

7 For the Western blot for p53 acetylation, the extracts were prepared using THP-1
8 cells treated with various concentrations of javamide-I and its O-methyl ester (0, 10, 20,
9 40) for 18 h. A same amount protein measured by Bio-Rad protein kit (Hercules, CA)
10 was loaded to the gel, also determined using anti-p53 antibodies (Cell Signal, Danvers,
11 MA, USA) . The levels of 53 acetylation was measured using acetylated-p53 antibodies
12 (Cell Signal, Danvers, MA, USA).

14 **Caspase-3/7 assay**

15 Caspase-3/7 activity was measured using a Caspase-3/7 assay kit (Promega,
16 Madison, WI), according to manufacturer's protocol. THP-1 cells were seeded in 96
17 well-culture dish and treated with javamide-I and its O-methyl ester (40 µM). After 0, 2,
18 4, 6, and 8 h incubation, the cells were collected and relative light units (RLU) output
19 was measured to determine caspase-3/7 activities.

21 **Cell death assay**

22 The apoptosis of THP-1 cells was monitored using PE Annexin V Apoptosis
23 Detection Kit I (BD Pharmingen, San Jose, CA). After the treatment of THP-1 cells with

1 javamide-I-O-methyl ester (0, 20, 40 μ M) for 18hr, the apoptosis of the cells were
2 monitored using the apoptosis kit containing PE annexin V and 7-Amino-Actinomycin
3 (7-AAD), according to manufacturer's protocol.

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5 **Statistic analysis**

6 Treatment effects were analyzed using the means for differences using One-way
7 ANOVA followed by Bonferroni's test. Differences were considered to be significant (p
8 < 0.05). Data points represent the mean $\pm$ SD (n = 4).

9

Results and discussion

Isolation of javamide-I, synthesis of javamide-I-O-methyl ester and NMR analysis.

The isolation and synthesis of javamide-I and its O-methyl ester (Fig. 1) was performed as described in "Materials and Methods". The final products were purified by HPLC (Waters, Milford, MA) and analyzed using NMR (supplementary data).

Sirt1/2/3 inhibition by javamide-I and its O-methyl ester.

The Sirt enzymes with highly conserved NAD⁺-binding domain are consisted of seven proteins (sirt1–7) (Bordo , 2013; Hall et al, 2013; Sebastián et al, 2012). All Sirt enzymes contain deacetylase activity which quickly alters protein activity and control a range of biological events including cell proliferation and death (Bordo , 2013; Hall et al, 2013; Sebastián et al, 2012; Zheng, 2013). Therefore, Sirt enzymes are considered as attractive target molecules in exploring valuable compounds able to prevent/treat abnormal metabolism, neurodegenerative diseases, aging and cancer (Zheng, 2013; Feldman et al, 2012). Seven Sirt enzymes are often found in specific cellular regions; sirt1, 6 and 7 (the nucleus), Sirt1 and 2 (the cytoplasm), and Sirt3, 4 and 5 (the mitochondria) (Hall et al, 2013; Sebastián et al, 2012). Therefore, in this study, the effects of javamide-I and its O-methyl ester on Sirt1 (nucleus), 2 (cytoplasm) and 3 (mitochondria) were investigated as described in "Materials and Methods". Javamide-I inhibited Sirt1 (IC₅₀ of 19 µM) stronger than Sirt2 (IC₅₀ of 104 µM) and 3 (IC₅₀ of 160 µM) (Fig. 2A) and similarly did the O-methyl ester; Sirt1 (IC₅₀ of 19 µM), Sirt2 (IC₅₀ of 115 µM) and Sirt3 (IC₅₀ of 174 µM) (Fig. 2B), suggesting that they may be able to

1 inhibit Sirt1 better than Sirt 2/3, which is different from javamide-II able to inhibit Sirt2
2 stronger than Sirt1 (Park, 2016).

4 ***Silico* and kinetic analysis**

5 *Silico* analysis was performed with javamide-I and the O-methyl ester against five
6 Sirt1 proteins (PDB_ID; 4IF6, 4IG9, 4I5I, 4KXQ, 4ZZH) in order to confirm the
7 observed Sirt1 inhibition. This analysis was carried out using ICM-pro as described in
8 "Material and Methods" . The analysis was performed using javamide-I or the O-methyl
9 ester to EX527 (a known Sirt1 inhibitor) on the accessible Sirt1 models (Table 1 and
10 Table 2). For *silico* analysis, each Sirt1 enzyme was separated into ligand and protein,
11 then experimental ligands (javamide-I, javamide-I-O-methyl ester, or EX527) were
12 introduced into the models. The analysis provided the lowest binding energy and scoring
13 values (Tables 1 and 2). The data showed that javamide-I and the O-methyl ester turned
14 out with better scores (more negative values) than EX527. Since they showed similar
15 *silico* data, only javamide-I with lowest binding energy was represented in Sirt1 complex
16 (PDB_ID; 4IF6) with two potential hydrogen bonds (green (S441) and blue (R466))
17 (Fig. 3A). Also a best-scored javamide-I to Sirt1 enzyme complex (4IF6) was depicted
18 with NAD⁺ (Fig. 3B) where javamide-I was coincided with NAD⁺ in a putative active
19 site. These data clearly suggest that javamide-I and the O-methyl ester may compete the
20 site with NAD⁺. To further define the inhibition pattern, kinetic analysis was carried out
21 to determine the K_i value. As shown in Fig. 4, javamide-I competitively inhibited Sirt1
22 against NAD⁺, with K_i = 20.1 μM, similarly javamide-I-O-methyl ester with K_i = 19.5
23 μM (The graph was not shown here). Both the *silico* and kinetic data indicated that

javamide-I and the O-methyl ester may be potent compounds inhibiting Sirt1 competitively.

p53 acetylation by javamide-I and its O-methyl ester.

One of well-known substrate for Sirt1 is p53 which is a potent transcription factor involved in cell cycle arrest and apoptosis via activating numerous genes possibly in the most cancer cell as well (Lane, 1992; Vousden and Prives, 2009). Therefore, we investigated the potential effects of javamide-I and its O-methyl ester on the acetylation of p53 in the THP-1 cells. The treatment with javamide-I and its O-methyl ester led to increase in the p53 acetylation, suggesting that the amides could inhibit the deacetylation of p53 in the cells (Fig. 5A and 5B). Although the two compounds were able to increase the p53 acetylation, the O-methyl ester was found to increase the acetylation in p53, significantly higher than javamide-I (Fig. 5B). This is quite unforeseen, because the *in vitro* assay data showed almost identical level of Sirt1/2/3 inhibition.

Caspase 3/7 induction by javamide-I and its O-methyl ester.

Since caspase 3/7 was often followed by p53 activation, we examined investigated the potential effect of on javamide-I and its O-methyl ester caspase 3. As shown in Fig. 6, the cells treated with the O-methyl ester showed significant activation of caspase 3/7 enzymes, compared to javamide-I ($p < 0.05$). The data suggest that the treatment of the O-methyl ester may increase p53 acetylation and induce caspase 3/7 activation better than javamide-I in the THP-1 cells.

Cell death by javamide-I-O-methyl ester.

Since javamide-O-methyl ester was able to induce caspase 3/7 activation better than javamide-I in the THP-1 cells, we investigated the effects of javamide-O-methyl ester on cell death. The cell death was clearly demonstrated in the THP-1 cells treated with the O-methyl ester (Fig. 7A, B and C), but not significant with javamide-I (data not shown here). All together, javamide-O-methyl ester may be a potent compound able to increase p53 acetylation, activate caspase 3/7 and induce cell death. Clearly, these data suggest that a small group alteration (O-methyl) in the carboxyl residue of javamide-I was able to increase the acetylation of p53 as well as induce caspase 3 activation better than parent javamide-I itself in the THP-1 cells, consequently led to cell death. Based on this discovery, we are currently synthesizing several ester analogues of javamide-I in order to determine potential effects of such alteration on the p53 acetylation, caspase 3 activation and cell death. Forthcoming studies may provide information concerning potential effects of the chemical modification in javamide-I on p53 acetylation, caspase 3 activation and their related cell death.

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Author contribution

The sole author

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Figure legends

Fig. 1. Chemical structures and HPLC chromatograms of javamide-I and its O-methyl ester. The isolation and chemical synthesis were performed as described in “Materials and Methods”. The chemical structures were verified using NMR, and the two peaks represent javamide-I and javamide-I-O-methyl ester.

Fig. 2. IC₅₀ values of javamide-I and its O-methyl ester against Sirt1. IC₅₀ was determined by the levels of Sirt1 inhibition javamide-I (A) and its O-methyl ester (B) at the range between 0.1 and 5000 μ M. Data points are shown with the mean $\pm$ SD (n = 4).

Fig. 3. *Silico* analysis of javamide-I into Sirt1. (A) Javamide-I to Sirt1 complex (4IF6) was presented, and two potential hydrogen bonds were presented in green (S441) and blue (R466). (B) Best-scored javamide-I to Sirt1 (4IF6) was presented with NAD⁺.

Fig. 4. Kinetic study of Sirt1 inhibition by javamide-I. Using Lineweaver-Burk plot, K_i values were determined. A is a control, and B and C with 25 and 50 μ M Javamide-I, respectively. The Sirt1 activity was determined using the substrate NAD⁺ at 200, 500, 1000, and 2000 μ M. Data points represent the mean $\pm$ SD (n = 3).

Fig. 5. Effects of javamide-I and its O-methyl ester on p53 acetylation. The p53 extracts were prepared using the THP-1 cells treated with various concentrations of javamide-I (A) and its O-methyl ester (B) (0, 10, 20, 40 μ M) in the presence of etoposide (10 μ M) for 18h, according to the kit’s manual. p53 blot was also provided as a control.

Fig. 6. Effects of javamide-I and its O-methyl ester on Caspase 3/7. THP-1 cells were pre-treated with javamide-I and its O-methyl ester (40 μ M). The capase-3/7 activation was measured at 0, 2, 4, 6, and 8 h. Data points represent the mean $\pm$ SD (n = 4).

Fig. 7. Effects of javamide-I-O-methyl ester on cell death. THP-1 cells were pre-treated with javamide-I-O-methyl ester (0 (A), 20 (B), and 40 (C) μ M). The cell death was measured at 18 h, using the apoptosis kit containing Annexin V-PE and 7-Amino-Actinomycin (7-AAD) (FL3).

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(B)

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Table 1. **Sirt1 *silico* data of javamide-I and EX527.** Energy and scores data were acquired using ICM-pro as described in "Material and Methods".

Javamide-I			EX527	
Sirt1 (PDB_ID)	Score	ΔE (Kcal/mol)	Score	ΔE (Kcal/mol)
4IF6	-56.4	-48.5	-16.7	-37.1
4IG9	-16.4	-17.5	-13.7	-23.5
4KXQ	-35.7	-49.3	-11.9	-37.4
4ISI	-15.4	-42.7	-41.7	-35.4
4ZZH	-28.6	-37.8	-11.5	-25.3
Average	-30.5	-39.1	-19.1	-31.7

Table 2. **Sirt1 *silico* data of javamide-I-O-methyl ester and EX527.** Energy and scores data were acquired using ICM-pro as described in "Material and Methods".

Javamide-I-O-methyl ester			EX527	
Sirt1 (PDB_ID)	Score	ΔE (Kcal/mol)	Score	ΔE (Kcal/mol)
4IF6	-51.2	-41.0	-16.7	-37.1
4IG9	-21.1	-20.6	-13.7	-23.5
4KXQ	-32.4	-43.5	-11.9	-37.4
4ISI	-21.5	-37.6	-41.7	-35.4
4ZZH	-25.4	-27.3	-11.5	-25.3
Average	-30.2	-34.0	-19.1	-31.7