New stereoisomeric derivatives of jasmonic acid generated by biotransformation with the fungus *Gibberella fujikuroi* affect the viability of human cancer cells

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ABSTRACT

Background: Several studies have shown that (-)-Jasmonic acid, (+)-7-iso-Jasmonic acid and its methyl ester, methyl jasmonate, have anti-cancer activity *in vitro* and *in vivo*, exhibiting selective cytotoxicity towards cancer cells. The degree of activity of these molecules is strongly related to their stereochemistry. The biotransformation of known compounds, natural or synthesized, related to interesting biological activities, generates new molecules displaying new improved properties compared with the original ones, increasing its value and providing new more effective products. Therefore, based on the above rationales and observations, in this work a biotransformation protocol to modify the chemical structure of the plant hormone jasmonic acid by using the fungus *Gibberella fujikuroi* was established.

Results: The three jasmonic acid derivatives obtained, 3(S)-Hydroxy-2(R)-(2Z-pentenyl)-cyclopentane-1(R)-acetic acid (1), 3(R)-Hydroxy-2(R)-(2Z-pentenyl)-cyclopentane-1(R)-acetic acid (2), 3-Hydroxy-2(S)-(2Z-pentenyl)-cyclopentane-1(S)-acetic acid (3), were tested for cell-growth inhibition activity towards the human cancer epithelial cell line, the oral squamous carcinoma cells (KB). The results obtained show that jasmonic acid derivatives (1-3) are active on human cancer cells examined in different concentration ranges, with IC50 value less than of 25 µM. The compound 3, with the same molecular structure of compounds 1 and 2, but with different stereochemistry, was more active confirming that the activity of jasmonate compounds is related to their stereochemistry and to substituents in the cyclopentane ring. In this study, we also tested the potential proapoptotic activity of compound 3, and our data suggest that it, as other jasmonate compounds, is able to trigger apoptotic death in cancer cells. This event may be correlated at an elevation of reactive oxygen species (ROS). Administration of N-acetylcysteine (NAC) prevented compound 3 cytotoxicity.

Conclusions: This work shows for the by first time the production of hydroxylated derivatives of JA by biotransformation. The activity observed of these compounds in cancer cells is higher than the observed with JA and is strongly related to its stereochemistry.

Keywords: apoptosis, biotransformations, cancer cells, cell vitality, *Gibberella fujikuroi*, jasmonic acid

INTRODUCTION

The interest in medicinal plants and their biologically active derivatives has increased in recent years, in relation to the possible development of drugs for several pathologies of relevant social impact.
(Heinrich and Gibbons, 2001). In fact, it is well known that medicinal plants are described and used in
respiratory disorders, in skin disorders, in cardiac diseases, in nervous and muscular disorders and in
mental health (Heinrich and Gibbons, 2001; Ankli et al. 2002). With respect to potential antitumor
activity, possible applications of natural compounds for cancer prevention and treatment have been
extensively described. Currently, over 60% of the anti-cancer agents in use are derived from natural
sources, i.e. plants, marine organisms and micro-organisms (Richardson, 2001; Wargovich et al. 2001;
Chang et al. 2002; Tatman and Mo, 2002; Ravelo et al. 2004; Cardile et al. 2005; Cragg and Newman,
2005; Aggarwal and Shishodia, 2006). Several studies have evidenced in recent years that (-)
Jasmonic acid (JA), (+)-7-iso-JA and its methyl ester, methyl jasmonate (MeJA), important members of
the jasmonate family, have anti-cancer activity in vitro and in vivo, exhibiting selective cytotoxicity
towards cancer cells while sparing normal blood lymphocytes, even when the latter were part of a
mixed population of leukemic and normal cells drawn from the blood of chronic lymphocytic leukemia
(PLL) patients (Flescher, 2007; Kniazhanski et al. 2008). Jasmonates, their precursors, or their
metabolites, are known to accumulate in higher plants (Wasternack, 2007) and fungi (Miersch et al.
1999a). These molecules, ubiquitous in the plant kingdom, constitute a group of bioactive fatty acid
derivatives that perform several important roles in growth, development, as well as biotic and abiotic
stress (Heinrich and Gibbons, 2001). This work a biotransformation protocol to modify the chemical structure of JA by using the fungus
G. fujikuroi, was established. The three JA derivatives obtained (1-3) (Figure 1) were tested for cell-growth
inhibition activity towards the human cancer epithelial cell line, the oral squamous carcinoma cells
(KB). Several biochemical parameters were tested, such as cell viability (MTT assay) and cell
membrane integrity (lactate dehydrogenase release) (Russo et al. 2006). In addition, the fragmentation
of genomic DNA determined by COMET assay and caspase-3 activity were examined (Russo et al.
2006). The possible induction of oxidative stress was evidenced by performing a fluorescent analysis of
intracellular reactive oxygen species (ROS) production (Russo et al. 2006).
MATERIALS AND METHODS

Chemicals

All reagents were of commercial quality and were used as received. For biotransformation experiments (±) Jasmonic acid was obtained from Sigma-Aldrich (St. Louis, MO, USA). Deuterated solvents and general chemical reagents were obtained from Merck S.A. (Santiago, Chile). For cell culture studies 3(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT), N-acetylcysteine (NAC) and β-nicotinamide-adenine dinucleotide (NADH) were obtained from Sigma Aldrich Co (St. Louis, USA). All other chemicals were purchased from Sigma Aldrich Co (St. Louis, USA) and GIBCO BRL Life Technologies (Grand Island, NY, USA).

Biotransformation experiments and chemical analysis

Microorganisms and culture conditions. The fungus G. fujikuroi was graciously provided by Dr. B. Fraga, Natural Products and Agrobiology Institute, CSIC, Canary Islands, Spain. The strain of G. fujikuroi was grown either on solid PDA (potato dextrose agar) or in liquid medium according to Fraga et al. 2000. G. fujikuroi was grown on surface culture in petri plates at 26°C for 5 days on PDA medium (25 ml per flask) comprising potato extract (4 g/L), glucose (20 g/L), and agar (15 g/L). The liquid medium used, according to the Fraga protocol modification, has the following composition: glucose (40 g/L), NH₄NO₃ (0.48 g/L), KH₂PO₄ (5 g/L), MgSO₄ 7H₂O (1 g/L), pH 5.0 and 2 mL of trace elements such as Co(NO₃)₂ (0.1 mg/L), (NH₄)Mo7O24 (0.1 mg/L) FeSO₄ 7H₂O (1 mg/L), CuSO₄ 5H₂O (0.15 mg/L), ZnSO₄ 7H₂O (1,61 mg/L), MnSO₄ 7H₂O (0.1 mg/L).

Biotransformation experiments. Figure 2 shows the protocol of the culture employed for the JA biotransformation by G. fujikuroi according to Fraga et al. (2000). In the macroculture, G. fujikuroi was grown in shake culture (220 rpm) at 26°C, light (4000-4500 lux), in conical flasks (250 mL) each containing sterile medium (50 mL). JA (in 0.2% ethanol) was equally distributed into each bottle and the incubation allowed to continue for further 11 days. Two culture control conditions were employed: i) G. fujikuroi growing on media without JA; and ii) JA mixed with medium in absence of G. fujikuroi, to corroborate JA stability by the medium. In both biotransformation and control samples, the culture conditions and the extraction methods employed were exactly the same. To obtain the compounds, the supernatant was adjusted to pH with aqueous acid chloride or sodium hydroxide to enhance neutral, acidic and basic extraction with ethyl acetate. The extracts were dried over sodium sulphate, the solvent was evaporated and the residues were separated and purified by CC and then identified by Thin Layer Chromatography (TLC).
Silica gel (Merck 200-300 mesh) was used for column chromatography and silica gel plates HF -254 for TLC. TLC spots were detected by heating after spraying with 25% H$_2$SO$_4$ in H$_2$O. The new identified spots were eluted from TLC by using petroleum ether/ ethyl acetate.

Low resolution mass spectra were recorded on a Shimadzu QP -2000 spectrometer at 70eV ionising voltage and are given as m/z (% rel. int.). NMR 1H-, 13C-(DEPT 135º and DEPT 90º), 2D COSY, 2D HSQC, and 2D HMBC spectra were recorded in CDCl$_3$ solutions and are referenced to the residual peaks of CHCl$_3$ at δ 7.26 and δ 77.0 ppm for 1H and 13C, respectively, on a Bruker Avance 400 Digital NMR spectrometer, operating at 400.1 MHz for 1H and 100.6 MHz for 13C. Chemical shifts are reported in δ ppm and coupling constants (J) are given in Hz.

### 3(S)-Hydroxy-2(R)-(2Z-pentenyl)-cyclopentane-1(R)-acetic acid (1)

$^1$H NMR: 5.53-5.38 (m, 2H, H-10 and H-9), 3.90 (dd, J = 5.6 and 11.2 Hz, 1H, H-3), 2.55 (dd, J = 4.9 and 15.2 Hz, 1H, H-6a), 2.29 (dd, J = 8.8 and 15.2 Hz, 1H, H-6b), 2.16-2.07 (m, 4H, H-11 and H-8), 1.94-1.84 (m, 3H, H-5, H-4 and H-1), 1.62-1.61 (m, 1H, H-4), 1.50-1.46 (m, 2H, H-5 and H-2), 0.97 (t, J = 7.5 Hz, 3H, H-12). $^{13}$C NMR: 40.2 (C-1), 53.9 (C-2), 78.7 (C-3), 33.5 (C-4), 30.3 (C-5), 39.8 (C-6), 173.8 (C-7), 29.2 (C-8), 126.9 (C-9), 133.8 (C-10), 20.8 (C-11), 14.4 (C-12); MS: 212 [M]+ < 1%, 211 (M+1, 12%), 155 (11%), 142 (17%), 134 (100%), 133 (25%), 119 (20%), 107 (16%), 95 (12%), 79 (20%).

### 3(R)-Hydroxy-2(R)-(2Z-pentenyl)-cyclopentane-1(R)-acetic acid (2)

$^1$H NMR: 5.52-5.37 (m, 2H, H-10 and H-9), 3.91 (dd, J = 5.2 and 10.2 Hz, 1H, H-3), 2.58 (dd, J = 4.8 and 15.2 Hz, 1H, H-6a), 2.34 (dd, J = 8.6 and 15.5 Hz, 1H, H-6b), 2.17-2.12 (m, 4H, H-11 and H-5), 2.09-1.87 (m, 3H, H-8, H-4 and H-1), 1.86-1.52 (m, 1H, H-4), 1.51-1.49 (m, 2H, H-8 and H-2), 0.96 (t, J = 7.4 Hz, 3H, H-12). $^{13}$C NMR: 39.5 (C-1), 53.6 (C-2), 78.4 (C-3), 33.2 (C-4), 30.0 (C-5), 39.8 (C-6), 178.2 (C-7), 29.0 (C-8), 126.5 (C-9), 133.7 (C-10), 20.6 (C-11), 14.2 (C-12); MS: 212 [M]+ < 1%, 194 (31%), 165 (20%), 153 (31%), 134 (100%), 125 (41%), 91 (54%), 83 (92%), 79 (88%).
New stereoisomeric derivatives of jasmonic acid

3(R or S)-Hydroxy-2(S)-(2Z-pentenyl)-cyclopentane-1(S)-acetic acid (3)

\(^1\)H NMR: 5.33-5.52 (m, 2H, H-10 and H-9), 4.22 (ddd, J = 1.7; 4.8 and 4.8 Hz, 1H, H-3), 2.55 (dd, J = 8.1 and 10.8 Hz, 1H, H-6), 2.05-2.22 (m, 7H, H-8, H-1, H-11 and H-5α), 1.87-1.91 (m, 1H, H-4), 1.42-1.44 (m, 1H, H-2), 1.25-1.32 (m, 1H, H-5β), 0.97 (t, J = 7.6 Hz, 3H, H-12). \(^13\)C NMR: 38.9 (C-1), 51.2 (C-2), 74.5 (C-3), 33.4 (C-4), 29.6 (C-5), 39.3 (C-6), 173.8 (C-7), 25.7 (C-8), 127.5 (C-9), 133.1 (C-10), 20.8 (C-11), 14.4 (C-12). MS: 211 ([M]-1 21%), 155 (48%), 142 (38%), 134 (100%), 119 (48%), 83 (30.5%), 79 (59%).

Study on tumor cell lines

Cell culture and treatments. KB cells (human epidermoid cells) were obtained from American Type Culture Collection (Rockville, MD, USA). Normal human non-immortalized buccal fibroblast cell line was kindly donated by Professor A. Simeone (IIGM, CNR, Naples, Italy).

KB cells (human epidermoid cells) were maintained in RPMI supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Normal human non-immortalised buccal fibroblast cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml fungizone. The cells were plated at a constant density to obtain identical experimental conditions in the different tests, thus to achieve a high accuracy of the measurements. In the MTT assay the cells were plated at 6 x 10^3 cells per well for human cancer cells, and at 2 x 10^4 cells per well for normal human non-immortalised buccal fibroblast cells, in a 96-well flat-bottomed 200 μl microplate. For other tests, the cancer cells were plated at 8 x 10^5 cells (2 ml) per 35 mm culture dish. After 24 hrs incubation at 37°C under a humidified 5% carbon dioxide to allow cell attachment, the cells were treated with different concentrations of JA and its derivatives (1-3), and incubated for 72 hrs under the same conditions.
Stock solution of the natural products was prepared in DMSO and the final concentration of this solvent was kept constant at 0.25%. Control cultures received DMSO alone.

**MTT bioassay.** MTT assay was performed as described previously (Russo et al. 2006). Briefly, cells were incubated at 37°C in a humidified 5% CO₂/95% air mixture and treated with different concentrations of JA and its derivatives (1-3) for 72 hrs. In some experiments, KB cells were treated by compound 3 (25 µM) with NAC (500 µM) for 72 hrs.

Four hours before the end of the treatment time, 20 µl of 0.5% 3(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) in phosphate buffer saline (PBS) were added to each microwell. Cells were washed once before adding MTT. After four hours of incubation at 37°C, the supernatant was removed and replaced with 100 µl of DMSO. The optical density of each well sample was measured with a microplate spectrophotometer reader (Digital and Analog Systems, Rome, Italy) at 550 nm.

**Lactate dehydrogenase (LDH) release.** Lactate dehydrogenase (LDH) activity was spectrophotometrically measured in the culture medium and in the cellular lysates at 340 nm by analyzing NADH reduction during the pyruvate-lactate transformation, as previously reported (Russo et al. 2006). Cells were lysed with 50 mM Tris-HCl + 20 mM EDTA pH 7.4 + 0.5% sodium dodecyl sulfate (SDS), further disrupted by sonication and centrifuged at 13,000 x g for 15 min. The assay mixture (1 ml final volume) for the enzymatic analysis contained: 33 µl of sample (5-10 µg of proteins) in 48 mM PBS pH 7.5 plus 1 mM pyruvate and 0.2 mM NADH. The percentage of LDH released was calculated as percentage of the total amount, considered as the sum of the enzymatic activity present in the cellular lysate and that in the culture medium. A Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) was used.

**DNA analysis by COMET assay.** The presence of DNA fragmentation was examined by single cell gel electrophoresis (COMET assay), according to Singh et al. (1991). Briefly, 0.8-1 x 10⁵ cells were mixed with 75 µl of 0.5% low melting agarose (LMA) and spotted on slides. The "minigels" were maintained in lysis solution (1% N-lauroyl sarcosine, 2.5 M NaCl, 100 mM Na₂EDTA, 1% Triton X-100, 10% DMSO, pH 10) for 1 hr at 4°C, and then denatured in a high pH buffer (300 mM NaOH, 1 mM Na₂EDTA, pH 13) for 20 min, and finally electrophoresed in the same buffer at 18 V for 45 min. At the end of the run, the "minigels" were neutralized in 0.4 M Tris-HCl, pH 7.5, stained with 100 µl of ethidium bromide (2 µg/ml) for 10 min and scored using a fluorescence microscope (Leica, Wetzlar, Germany) interfaced with a computer. Software (Leica-QWIN) allowed to assess the quantitative and qualitative extent of DNA damage by measuring: a) tail length (TL), intensity (TI) and area (TA); b) head length (HL), intensity (HI) and area (HA). Finally, the program using these parameters calculates the level of DNA damage as: i) the percentage of the fragmented DNA (TDNA), and ii) tail moment (TMOM). The tail moment is defined as the product of the percentage of DNA in the tail of the comet and TD value.

### Table 2. Lactate dehydrogenase (LDH) release in KB cells untreated and treated with jasmonic acid (JA) and compounds 1-3 at different concentrations for 72 hrs.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>KB cells % LDH released</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td>Compound JA</td>
<td></td>
</tr>
<tr>
<td>12.5 µM</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>25 µM</td>
<td>7.1 ± 0.7</td>
</tr>
<tr>
<td>Compound 1</td>
<td></td>
</tr>
<tr>
<td>12.5 µM</td>
<td>6.3 ± 0.7</td>
</tr>
<tr>
<td>25 µM</td>
<td>6.6 ± 0.3</td>
</tr>
<tr>
<td>Compound 2</td>
<td></td>
</tr>
<tr>
<td>12.5 µM</td>
<td>6.2 ± 0.4</td>
</tr>
<tr>
<td>25 µM</td>
<td>3.9 ± 0.7</td>
</tr>
<tr>
<td>Compound 3</td>
<td></td>
</tr>
<tr>
<td>12.5 µM</td>
<td>5.5 ± 0.6</td>
</tr>
<tr>
<td>25 µM</td>
<td>4.3 ± 0.2</td>
</tr>
</tbody>
</table>

The results are expressed as percentage of LDH released into the cell medium with respect to total LDH. Each value represents the mean ± SD of three experiments, performed in quadruplicate.
which is obtained calculating the distance between the center of mass of the comet head and the center of mass of the tail. The percentage of DNA in the comet tail was calculated as the rate of the fluorescence intensity in the comet tail relative to the total fluorescence; 100 randomly selected cells were analyzed per sample.

**Activity of caspase-3.** The activity of caspase-3 was determined by using the Caspase colorimetric assay Kit (SIGMA RBI St. Louis, USA). This assay measures the cleavage of a specific colorimetric caspase substrate, acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA). pNA (p-nitroaniline) is released from the substrate upon cleavage by caspase. Free pNA produces a yellow colour that is monitored by a Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) at λ = 405 nm. The caspase-3 activity was measured in cell lysates. The cell pellets were incubated at 4°C for 20 min with lysis buffer containing 50 mM HEPES (1-piperazineethane sulfonic acid, 4-(2-hydroxyethyl)monosodium salt), pH 7.4, 5 mM CHAPS (3[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid), 5 mM DTT (1,4 dithio-DL-threitol). The lysed cells were centrifuged at 16,000 x g for 15 min at 4°C, and the supernatants were analyzed immediately according to the analysis procedure described in the manufacturer’s protocol.

The total protein content, used to reflect cell number and measured according to Bradford (1976), was evaluated for each sample, and the results are reported as OD_{405} nm/mg protein and compared to relative control.

**Reactive oxygen species assay.** Reactive oxygen species (ROS) determination was performed by using a fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA), as previously described (Russo et al. 2006). DCFH-DA, diffuses through the cell membrane, and it is enzymatically hydrolysed by intracellular esterases and oxidized to the fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS. The intensity of fluorescence is proportional to the levels of intracellular oxidant species. 100 μl of 100 μM DCFH-DA, dissolved in 100% methanol was added to the cellular medium where the acetate group is not hydrolyzed, and the cells were incubated at 37°C for 30 min. After incubation, cells were lysed and centrifuged at 10,000 g for 10 min. The fluorescence (corresponding to the radical species-oxidized 2',7'-dichlorofluorescein, DCF) was monitored spectrofluorometrically using a Hitachi F-2000 spectrofluorimeter (Hitachi, Tokyo, Japan): excitation 488 nm, emission 525 nm. The total protein content, measured according to Bradford (1976) was evaluated for each sample, and the results are reported as fluorescence intensity/mg protein and compared to relative control.
Statistical analysis

Results were analyzed using one-way ANOVA followed by Dunnett’s post-hoc test for multiple comparisons with control. All statistical analyses were performed using the statistical software package SYSTAT, version 9 (Systat Inc., Evanston IL, USA).

RESULTS AND DISCUSSION

Biotransformation experiments and chemical analysis

The biotransforming capacity of *G. fujikuroi* mainly on terpene compounds has been well described in the literature (Fraga et al. 2000; Fraga et al. 2003). One of the best characterized biotransformation systems using *G. fujikuroi* is the chemical modification of some precursors of the plant hormone gibberellins (Barrero et al. 2001; Fraga et al. 2004; Fraga et al. 2007). The fungus shows the presence of enzymes involved in the biosynthetic pathway of such type of diterpenoids which might be responsible for the chemical modification of the gibberellin structure (Tudzynski et al. 2003; Malonek et al. 2004; Tudzynski, 2005). Also described has been the ability of this fungus for producing the plant hormone jasmonic acid in small amounts and mainly being detected in the supernatant of the mediaculture (Miersch et al. 1992). This suggests the occurrence of enzymatic activities allowing JA production and a potential machinery able for transforming the chemical structure of this molecule. Indeed, in this work, growth of the fungus *G. fujikuroi* in a culture media containing JA and analysis of the different extracts by Thin Layer Chromatography (TLC), demonstrated the presence of new bands different to the original JA. Their extraction under neutral conditions (pH 6.5-7.0) presented more compounds than any other extracts obtained under basic or acidic conditions. The analysis by TLC showed the presence of new spots with different polarities compared to JA (data not shown). Purification of these spots with increasing gradient solutions of petroleum ether: ethylacetate from 10:3 to 2:8 by silica gel columns permitted the separation of three compounds denominated 1, 2, 3. The 1H-NMR spectra of these fractions demonstrated that all of them displayed a pattern of signals similar to JA. These three compounds (1, 2, and 3) also showed 1H-NMR spectra with similar chemical shifts among them. In addition, all three compounds presented a new signal corresponding, by their chemical displacement, to a hydrogen attached to a carbon (C-3) together with a hydroxyl group. Furthermore,
the signal of the carbon of the ketone group in the $^{13}$C-NMR spectrum was not observed. The small
difference existing in the displacement of this hydrogen (H-3) between the three derivatives suggested
that the carbonyl group was reduced to a hydroxyl group, but with a stereochemistry different to that
present in the original JA molecule. In fact, these generated compounds (1, 2 and 3) correspond to the
same molecule with different stereochemistry among them.

The hydroxylated molecules generated here (1, 2 and 3) by means of the enzymatic action of G.
fujikuroi correspond to the molecule cucurbitic acid (CA) and some of its stereoisomers, a metabolic
derivative of JA, whose presence has been detected in both higher plants and fungi in low contents
(Dathe et al. 1991; Flescher 2007). The chemicals synthesis of some isomers of CA has also been
reported (Dathe et al. 1991; Miersch et al. 1999a; Seto et al. 1999; Flescher 2007) and their structure-
function relation in plants has been characterized, suggesting that the essential structural factors of this
type of compound pointed to the cis configuration of C-1 and C-2 substituents, the free hydroxyl group
at the C-3 position, alkenyl or alkyl group at the C-2 position (Seto et al. 1999). Searching the literature
no reports of CA synthesis and stereoisomers of this compound by biotransformation could be found
and no reports have been found that account for the antitumor effect of CA and its stereoisomers.
Previous reports on JA biotransformation using the fungus Aspergillus niger only mention specific
hydroxylations in position C-11 on the jasmonate molecule (Miersch et al. 1999c).

The isomerized product was obtained by biotransformation. The structural determination of 1, 2 and
3 was mainly accomplished by $^1$H, $^{13}$C, DEPT-135, gs-2D (HHCOSY, HSQC and HMBC) NMR
techniques and compared with those previously reported (Seto et al. 1999).

In Figure 1 the chemical structures produced by biotransformation of JA with G. fujikuroi are shown. As
results of the biotransformations, three hydroxylated compounds of JA were obtained (1, 2 and 3).

The spectra of the derivatives of the JA biotransformation are product of enzymatic activities of this
fungus that have not yet been studied nor reported. Relative to the hydroxylated compounds, the
existence of reduction reactions of the ketone group in the cyclopentane ring of the original molecule is
foreseen due to a possible reductase activity. Important information derived from these derivatives
could be drawn, since they are a proof for the existence of stereospecific enzymes not yet informed for
G. fujikuroi. Relative to the isomerized derivative corresponding to an isomerization product of the acid
hydrogen (H-2) of JA, it is not known which enzyme could be responsible for generating this
modification; autoisomerization is discarded since in the liquid control medium in presence of JA
(without fungus) no modification of any type of the compound was observed.

Table 3. Comet assay of genomic DNA in KB cells untreated and treated with compound 3 at different
concentrations for 72 hrs. Stock solution of compounds was prepared in DMSO and the final concentration of this
solvent was kept constant at 0.25%. Control cultures received DMSO alone.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>KB cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>93 ± 3.4</td>
</tr>
<tr>
<td>Compound 3</td>
<td>1096 ± 18*</td>
</tr>
<tr>
<td>6.25 µM</td>
<td>1320 ± 36*</td>
</tr>
<tr>
<td>12.5 µM</td>
<td>2765 ± 31*</td>
</tr>
</tbody>
</table>

*TMOM= tail moment expressed as the product of TD (distance between head and tail) and TDNA. The values are the
mean ± SD of three experiments performed in quadruplicate. *Significant vs. control untreated cells (p < 0.001).

Study on tumor cell lines

Since antiproliferative screening models in vitro provide important preliminary data to help select
compounds with potential antineoplastic properties for future study, JA and its derivatives (1-3) were
tested in vitro for their potential human tumour cell growth inhibitory effect on KB cells, using MTT
assay, a non-radioactive, fast and economical assay widely used to quantify cell viability and
proliferation. MTT is a yellow water-soluble tetrazolium salt. Metabolically active cells are able to
convert the dye to water-insoluble dark blue formazan by reductive cleavage of the tetrazolium ring.
The results, summarized in Table 1, show that JA and its derivatives (1-3), such as other jasmonate
compounds, that are specifically active on several types of cancer cells in different concentration
ranges (Flescher, 2007), exhibited an inhibitory effect on the human cancer cells examined with different IC50 value. Interestingly, the compound 3, with the same molecular structure of compounds 1 and 2, but with different stereochemistry, was more active than jasmonic acid against human cancer epithelial cell line, KB cells. These results confirm that the activity of jasmonate compounds is related to their stereochemistry and to substituents in the cyclopentane ring (Miersch et al. 1999b). In addition, the JA derivatives, also at 100 μM concentration, revealed no cytotoxic effect against normal human buccal fibroblast cells (BFC) (Figure 3), considered a useful model to evaluate the cytotoxic effects of carcinogens (Galvano et al. 2002) and the tumour-specific cytotoxicity of potential antineoplastic agents (Sekine et al. 2007).

There are many reports that show that members of the plant stress hormones family, and some of their synthetic derivatives, exhibit anti-cancer activity in vitro and in vivo (Fingrut and Flescher, 2002; Flescher, 2007).

Likewise, it is reported the synthesis of numerous and varied series of derivatives of MeJA, predominantly halogenated (-Cl,-Br,-F) structures, which similar to JA and its derivatives, they exhibit interesting and in some cases potent anti-neoplastic properties (Ishii et al. 2004; Reischer et al. 2007; Park et al. 2010; Zhao et al. 2010), but it is noteworthy that in each of the proposed series does not have reported the production of derivatives with keto group reduced and the potential inhibition of cell growth observed with these series of compounds does not exceed the IC50 obtained with the derivatives reported here.

Qian et al. 2004 reported that synthetic hydroxyl-containing jasmonates may induce different levels of plant defense responses, which are associated with different levels of enhanced metabolic activity for secondary metabolite biosynthesis. This stimulating activity can be explained by the effect of the estereocconfiguration and the optimal lipophilicity and the number of hydroxyl groups. Following the same line of comparison between the effects caused by plant stress hormones and their derivatives in plants under stress and tumour cells would be assumed that the influence on lipophilicity provided by hydroxyl groups present in jasmonates derivatives reported in plants, is also an important factor in the antitumor effect observed in cancer cells; This structural difference could be making the difference in the potential of its activity between jasmonic acid (JA) and its reduced derivative, cucurbitic acid (CA).

Cells can die in two different ways: necrosis and apoptosis. Necrosis is caused by outside damage, recognized by destruction of plasma membrane and intracellular organelles and molecules. In
New stereoisomeric derivatives of jasmonic acid

apoptosis, biochemical and morphological events are usually organized in a cascade of very specific and controlled steps. The beginning of apoptosis is marked by chromatin condensation that occurs in parallel to the shrinking of the cell. Fragmentation of the nucleus, transformation of the cell surface, and complete splitting of the cell contents to apoptotic bodies attached to the membrane, accompany those changes. One of the important features of apoptosis, in most cell lines, is DNA fragmentation, catalyzed by endogenic endonucleases. Final events of apoptosis include activation of specific cytoplasmic proteases named caspases. In mammalian cells, caspases are activated in a cascade, resulting in inappropriate activation or fast damage to structural proteins, as well as to important signaling processes, homeostasis and repair enzymes. Caspase-3 is essential for a number of morphological and biochemical events associated with apoptosis (Shi, 2002). Alterations in apoptotic cell death can lead to a loss of growth control, and thus play a major role in the process of tumorigenesis. Defects of apoptotic pathways influence also drug resistance, and because of these defects chemotherapy often fails (Johnstone et al. 2002). The induction of apoptosis in tumor cells is considered very useful in the management and therapy of cancer (Johnstone et al. 2002). Synthesis or modification of known drugs continues as an important aspect of research. There is a continued need for new templates to be used in the design of potential chemotherapeutic agents. Significantly, natural products are providing such templates. Recent studies on tumor inhibitory compounds of plant origin have yielded an impressive array of novel structures (Ravelo et al. 2004; Cragg and Newman, 2005; Aggarwal and Shishodia, 2006). Despite the structural differences between members of the family of jasmonates and their natural and synthetic derivatives, in general, the mechanism of antitumor action lies in the induction of apoptosis, DNA fragmentation, and increased caspase activity. There are many works that report that cellular stressors induce inhibition of cell proliferation and cell death. Salicylic acid, JA and its methylated derivative (MeJA) are plant stress hormones that activate cellular responses, including cell death, to diverse stress conditions in plants, and also can suppress the proliferation or cause apoptosis in various cancer cells without affecting the normal cells (Flescher, 2007). This data support the potential use of plant stress hormones as selective anti-cancer agents and opens the possibility to use more powerful new derivatives.

Consistent with this approach, in this study we tested the potential proapoptotic activity of compound 3, and our data suggest that it, as other jasmonate compounds (Flescher, 2007), is able to trigger apoptotic death in cancer cells. In fact, no statistically significant increase in LDH release (Table 2), a marker of membrane breakdown, correlated to a high DNA fragmentation (Table 3), evidenced by the Comet assay, a versatile tool to distinguish apoptotic from necrotic cells (Russo et al. 2006), was observed in cancer cells treated with this compound at 6.25, 12.5 and 25 µM concentrations. The hypothesis of apoptosis induction in our experimental conditions was reinforced by a significant (p < 0.001) increase of the caspase-3 enzyme activity (Figure 4).
ROS have been reported to be involved in cell death induced by a variety of stimuli and different antitumoral agents. Analysis of MJ-induced death in A549 human lung adenocarcinoma cells revealed that MJ-induced apoptosis is correlated to generation of reactive oxygen species (Flescher, 2007). We therefore examined whether cell death induced by compound 3 may be correlated at an elevation of ROS. To assess changes in intracellular ROS levels, we employed an oxidation-sensitive fluorescent probe DCFH-DA. DCFH-DA can be taken up into cells, and then oxidized by ROS to its fluorescent derivative DCF. As supposed, we found that the DCF fluorescence increased in a concentration-dependent manner in the human cancer cells exposed to compound 3 (Figure 5). To confirm the role of ROS in compound 3-induced cell death, KB cells were treated by compound 3 (25 μM) with the antioxidant NAC (500 μM) for 72 hrs, following which cell viability was determined. NAC control group did not show any significant changes in growth inhibition compared with untreated controls (Figure 6). Treatment with NAC significantly protected cancer cells against the growth inhibitory effect of compound 3 (Figure 6). This suggests that increased ROS generation is essential in compound 3-mediated cell death.

CONCLUDING REMARKS

Although the compound 3 absolute configuration of carbon five remains to confirm, and of course further studies are needed, taken together, the results shown in this work demonstrated that the production of compounds obtained by the biotransformation of JA with G. fujikuroi may offer a further impulse to the development of the studies towards the optimization of the production in culture media, as well as to be a source for the synthesis of compounds with more potential antitumor activity.

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REFERENCES


STASWICK, P. (2009). The tryptophan conjugates of jasmonic and indole-3-acetic acids are endogenous auxin inhibitors. *Plant Physiology*, vol. 150, no. 3, p. 1310-1321. [CrossRef]


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