

# AKR1C Isoforms Represent a Novel Cellular Target for Jasmonates alongside Their Mitochondrial-Mediated Effects

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## Abstract

Members of the aldo-keto reductase (AKR) superfamily, particularly the AKR1C subfamily, are emerging as important mediators of the pathology of cancer. Agents that inhibit these enzymes may provide novel agents for either the chemoprevention or treatment of diverse malignancies. Recently, jasmonates, a family of plant stress hormones that bear a structural resemblance to prostaglandins, have been shown to elicit anticancer activities both *in vitro* and *in vivo*. In this study, we show that jasmonic acid (JA) and methyl jasmonate (MeJ) are capable of inhibiting all four human AKR1C isoforms. Although JA is the more potent inhibitor of recombinant AKR1C proteins, including the *in vitro* prostaglandin F synthase activity of AKR1C3, MeJ displayed greater potency in cellular systems that was, at least in part, due to increased cellular uptake of MeJ. Moreover, using the acute myelogenous leukemia cell lines HL-60 and KG1a, we found that although both jasmonates were able to induce high levels of reactive oxygen species in a dose-dependent fashion, only MeJ was able to induce high levels of mitochondrial superoxide (MSO), possibly as an epiphenomenon of mitochondrial damage. There was a strong correlation observed between MSO formation at 24 hours and reduced cellularity at day 5. In conclusion, we have identified AKR1C isoforms as a novel target of jasmonates in cancer cells and provide further evidence of the promise of these compounds, or derivatives thereof, as adjunctive therapies in the treatment of cancer. [Cancer Res 2009;69(11):4769–75]

## Introduction

It is becoming increasingly clear that human aldo-keto reductases (AKR) of the AKR1C family are intimately linked with cancer biology. For example, AKR1C enzymes activate xenobiotic polyaromatic hydrocarbon (PAH) proximal carcinogens *in vitro*. The *in vivo* importance of this is strongly supported by observations that AKR1C3 polymorphisms modulate risk for lung cancer due to exposure to PAH-rich coal combustion emissions (1). Furthermore, AKR1C3 polymorphisms have been shown to modulate risk of other cancers, including childhood leukemias, diffuse large B-cell lymphoma, and carcinomas of the prostate and bladder (2–5). Separately, it has been recently shown that AKR1C3 inactivates the anticancer drugs doxorubicin and irinotecan (6). Thus,

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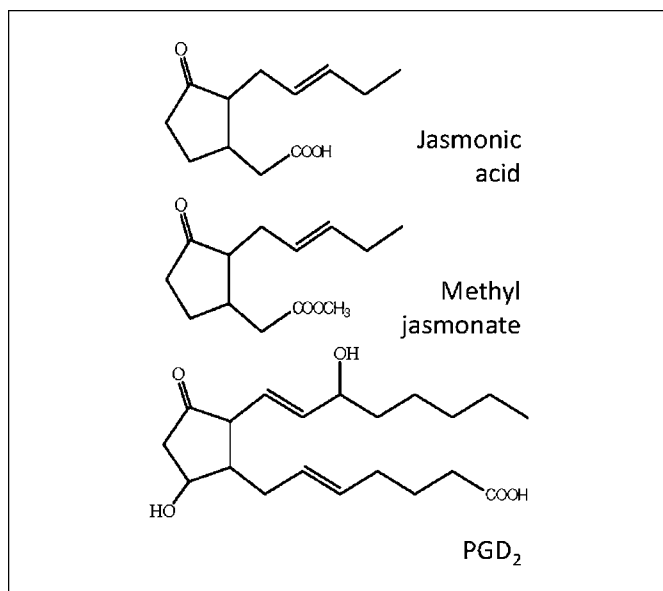
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the ability of AKR1C3 to use xenobiotics as substrates is linked with both the etiology of cancers and therapeutic resistance.

We have previously shown that AKR1C3 regulates myeloid cell differentiation. Treatment of HL-60 cells with the AKR inhibitors indomethacin or medroxyprogesterone acetate enhances both neutrophil and monocyte differentiation (7, 8), and reciprocally, overexpression of AKR1C3 suppresses HL-60 differentiation (8, 9). More recently, we have shown that knockdown of AKR1C3 in K562 cells results in erythroid differentiation (10). We have therefore proposed AKR1C3 as a novel regulator of myeloid cell differentiation and a potential new therapeutic target in leukemia. Similarly, others have identified AKR1C3 as a possible target in prostate and breast carcinoma (11, 12). Equally, other studies have identified AKR1C2 and AKR1C1 as potential targets for the treatment of prostate, breast, lung, and bladder carcinomas (12–15). Thus, the availability of AKR1C-selective inhibitors would be of value in the chemoprevention of cancers, in use as adjuvant therapies in combination with established chemotherapeutics and as direct therapeutic interventions in their own right. In recognition of this, several laboratories have undertaken structural studies of AKR1C3 bound to inhibitors and substrates and embarked on the identification of lead compounds for drug development (16–21).

An established approach to identifying new drugs is to identify natural products from plants with the desired activities (22). Several structurally and functionally diverse plant hormones, including auxins (23), cytokinins (24), and jasmonates (25, 26), have been shown to possess anticancer properties. Jasmonates are fatty acid-derived cyclopentanones that are structurally similar to prostaglandins in metazoans. Members of the jasmonate family include jasmonic acid (JA) and methyl jasmonate (MeJ; Fig. 1), which have been shown to play major roles in defense against insects and disease, as well as during plant growth and development (27). The cytotoxicity of jasmonates has been shown *in vitro* against cancer cell lines derived from colon, breast, lung, prostate, melanoma, and glioma as well as leukemia cell lines and primary chronic lymphocytic leukemia samples (25, 28–30). Oral administration of jasmonates was also found to improve the survival of mice that had been engrafted with a murine T lymphoma cell line (25). Furthermore, combinatorial treatment of leukemia cell-bearing mice with MeJ and the anthracycline doxorubicin increased survival of mice compared with those treated with either agent alone (28).

A key activity of AKR1C3 not shared by the other AKR1C enzymes is the metabolism of prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) into 9 $\alpha$ ,11 $\beta$ -prostaglandin F<sub>2 $\alpha$</sub>  (9 $\alpha$ ,11 $\beta$ -PGF<sub>2 $\alpha$</sub> ; ref. 31). PGD<sub>2</sub> spontaneously dehydrates to J-series prostaglandins, which culminates in the formation of 15-deoxy- $\Delta^{12,14}$ PGJ<sub>2</sub> (15dPGJ<sub>2</sub>). This highly bioactive prostaglandin is a peroxisome proliferator-activated receptor- $\gamma$  ligand, inhibits nuclear factor- $\kappa$ B, and elevates reactive oxygen species (ROS; refs. 32–35). Therefore, it is inhibition of the



**Figure 1.** Jasmonates and prostaglandins are structurally similar. Shown are chemical structures of JA (*top*), MeJ (*middle*), and PGD<sub>2</sub> (*bottom*).

PGD<sub>2</sub> 11-keto reductase activity of AKR1C3, which we have shown, that results in elevated 15dPGJ<sub>2</sub> and promotes differentiation of myeloid leukemia cell lines.<sup>1</sup>

Due to the structural similarity of jasmonates and prostaglandins (Fig. 1), we wished to investigate whether AKR1C3 represents a novel target for the antitumor activities of jasmonates. Using both recombinant protein and cell-based assays, we show that both MeJ and JA inhibit members of the AKR1C subfamily, including AKR1C3. Furthermore, we show that the treatment of the acute myelogenous leukemia (AML) cell lines HL-60 and KG1a with both JA and MeJ results in the dose-dependent production of ROS, specifically mitochondrial superoxide (MSO), which seems to correlate with killing of these cell lines.

## Materials and Methods

Unless otherwise stated, reagents were purchased from Sigma.

**Cloning, production, and purification of recombinant AKR1C proteins.** DNA encoding full-length AKR1C1, AKR1C2, and AKR1C4 (accession numbers NM\_001353, NM\_001354, and NM\_001818) was obtained from Gene Service Ltd. and cloned into pET28b (Novagen). Plasmids were transformed into *Escherichia coli* BL21(DE3) cells to produce NH<sub>2</sub>-terminally His<sub>6</sub>-tagged recombinant proteins. These were expressed, extracted, and purified along with recombinant AKR1C3 (rAKR1C3) as previously described (19).

**Enzymatic assays of phenanthrenequinone reduction.** Phenanthrenequinone was dissolved in acetonitrile to make a 5 mol/L stock and subsequently diluted further in acetonitrile. Assays for all four human rAKR1C enzymes were performed using 15 μg recombinant protein in 50 mmol/L potassium phosphate buffer (pH 6.5) containing β-NADPH (15 μmol/L) and between 1.25 and 20 μmol/L of phenanthrenequinone at 35°C. JA or MeJ was used at concentrations of 20, 100, or 400 μmol/L. For all assays, acetonitrile and ethanol levels were constant at 2% (v/v) each. Initial

velocities were determined by measuring the change in absorbance of pyridine nucleotide at 340 nm ( $\epsilon = 6,270 \text{ mol/L}^{-1} \text{ cm}^{-1}$ ) in a 1 mL volume. The  $K_i$  and type of inhibition were calculated using the Visual Enzymics program (Softzyomics).

**Cells.** HL-60 and KG1a cells were maintained in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum and 100 units/mL penicillin and 100 μg/mL streptomycin (both from Invitrogen Ltd.).

**Assessment of jasmonate uptake into cells.** To ensure that no differential extraction of jasmonates was observed, extractions of intracellular JA and MeJ was performed alongside samples containing equal quantities of either jasmonate (2 μmol of each) alone or combined.

To assess uptake of jasmonates into HL-60 and KG1a cells,  $1 \times 10^7$  cells were cultured in 24-well plates in a final volume of 1 mL RPMI 1640 for 15 min at 37°C in the presence of either 10 mmol/L JA or MeJ before harvesting tubes and washing in PBS. Following centrifugation, the supernatant was aspirated and cell pellets were lysed by the addition of 500 μL of 50% (v/v) aqueous methanol and vortexed for 1 min before microfuge centrifugation for 10 min at 13,000 rpm. The resulting supernatants were acidified by addition of 80 μL concentrated HCl with 1 mL dichloromethane on ice. Tubes were vortexed for 1 min before centrifugation at  $374 \times g$  for 10 min. Following the removal of the upper aqueous layer, the dichloromethane phase was dried at 55°C under a stream of nitrogen before being redissolved in 50 μL of 50% (v/v) aqueous acetonitrile.

Extracts were analyzed by high-performance liquid chromatography (HPLC) using a 5-μm C18 column (Grace Davison Discovery Sciences). Jasmonates were separated using a gradient from 2% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid (TFA) to 98% (v/v) acetonitrile, 0.1% (v/v) TFA gradient. Cell-free extracts (5 μL) and 20 μL cell extracts were injected onto the column. Relative amounts of jasmonates were assessed at 210 nm. Peak areas were calculated by Chromeleon Client software (Dionex).

**Analysis of PGD<sub>2</sub> metabolism by TLC.** Either  $2 \times 10^7$  pretreated cells or 3 μg of recombinant protein were incubated in a final volume of 200 μL PBS in 96-well plates with 0.2 μCi of [<sup>3</sup>H]PGD<sub>2</sub> (Amersham) and the addition of the appropriate treatment. Prostanoids were extracted from the supernatant and separated using TLC as previously described (36). Once dry, TLC plates were scanned using a Bioscan AR-2000 plate reader. The migration of prostanoids was identified by their comigration with known standards as revealed by incubation in iodine vapor.

**Cell treatments.** JA and MeJ were dissolved in ethanol to make 1 mol/L stocks and stored at -20°C. For use in viability and flow cytometry assays, cells were seeded at  $2.5 \times 10^5$ /mL as 4 mL cultures in 25 cm<sup>2</sup> flasks and treated with a serial dilution of each agent (a range between 4 and 0.25 mmol/L), with carrier added to the control cultures. To prevent cells from becoming confluent, cells were reseeded on days 2 and 4 at  $2.5 \times 10^5$ /mL in the presence of the relevant concentration of jasmonates. For use in PGD<sub>2</sub> turnover assays, cells were seeded at  $5 \times 10^5$ /mL as 8 mL cultures in 25 cm<sup>2</sup> flasks and treated overnight with either 1 mmol/L JA, 1 mmol/L MeJ, or carrier control.

**Cell viability assay.** At day 5, duplicate 200 μL volumes of each treatment were transferred from the 25 cm<sup>2</sup> flasks to a 96-well plate before the addition of 20 μL CellTiter-Blue Cell Viability Assay reagent (Promega). Following mixing, the plate was incubated for 4 h at 37°C, and subsequently, fluorescence was measured at an excitation of 530/25 nm and emission of 595/35 nm on a KC4 fluorometer (Bio-Tek Instruments). Fluorescence bears a direct relationship to cell viability in cultured cells. Background readings were assessed using wells containing medium alone, and these measurements were subtracted from readings obtained for samples. To account for differences in readings due to the different feeding regimens on days 2 and/or 4, results were adjusted accordingly to account for cells that were removed from the cultures.

**Assessment of mitochondrial depolarization and generation of ROS and MSO.** All analyses were carried out by flow cytometry on a FACSCalibur using CellQuest Pro (Becton Dickinson) software. Mitochondrial inner membrane potential was assessed using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1), which was dissolved at 5 mg/mL in DMSO and stored at -20°C and for experimental

<sup>1</sup>F.L. Khanim and colleagues. Rational redeployment of bezafibrate and medroxyprogesterone acetate as novel therapy for elderly and relapsed acute myeloid leukaemia, submitted for publication.

use was further diluted to 1 mg/mL in PBS. At 24 h after treatment, 200  $\mu$ L of cell suspension were diluted to 1 mL with warm PBS with the addition of JC-1 at a final concentration of 10  $\mu$ g/mL and incubated at 37°C, 5% CO<sub>2</sub> for 10 min. Subsequently, the samples were washed with 1 mL PBS, resuspended in 300  $\mu$ L PBS, and kept on ice until analyzed.

Carboxy-H<sub>2</sub>DCFDA (Molecular Probes) is a membrane-permeable compound that is used to assess all cellular ROS. It was dissolved in DMSO to yield a 2000 $\times$  stock and stored under nitrogen at -20°C. Immediately before use, a 2 $\times$  (10  $\mu$ mol/L) working dilution was made in warm PBS. At 24 h after treatment, 200  $\mu$ L of cell suspension were mixed with 200  $\mu$ L of the working stock and incubated at 37°C, 5% CO<sub>2</sub> for 40 min before analysis by flow cytometry (emission wavelength, 517–527 nm).

MitoSOX Red (Molecular Probes) was used to assess the presence of MSO. Before use, MitoSOX Red was dissolved in DMSO to yield a 5 mmol/L stock and subsequently diluted to a working concentration of 5  $\mu$ mol/L in warm PBS. At 24 h after treatment, 200  $\mu$ L of cell suspension were centrifuged, the supernatant was removed, and 200  $\mu$ L of working stock were added followed by incubation at 37°C for 10 min before analysis by flow cytometry (emission wavelength, 580 nm).

## Results

**Jasmonates inhibit AKR1C activity.** Due to the structural similarities between jasmonates and PGD<sub>2</sub>, we hypothesized that these plant hormones may be able to inhibit AKR1C3. Although not a natural substrate, phenanthrenequinone represents a useful tool to study the inhibitory effects of compounds against AKR1C isoforms because most, if not all, members of this subfamily readily catalyze its reduction (18).<sup>2</sup> We therefore investigated the inhibition by jasmonates of the NADPH-dependent reduction of phenanthrenequinone by rAKR1C proteins. Both MeJ and JA competitively inhibited rAKR1C3, with JA displaying greater potency than MeJ with a  $K_i$  greater than 5-fold lower (Table 1). Similarly, both JA and MeJ inhibited rAKR1C1 and rAKR1C2, but only JA was found to be able to sufficiently inhibit rAKR1C4 at the doses tested here. Importantly, JA displayed greater inhibition for all four rAKR1C enzymes than MeJ.

**Jasmonates inhibit AKR1C3 PGD<sub>2</sub> 11-keto reductase activity.** To identify whether jasmonates could also inhibit the PGD<sub>2</sub> 11-keto reductase activity of AKR1C3, we performed a series of enzyme assays using rAKR1C3 and [<sup>3</sup>H]PGD<sub>2</sub> and assessed inhibition following separation of the products using TLC (Fig. 2A). The production of 9 $\alpha$ ,11 $\beta$ -PGF<sub>2 $\alpha$</sub>  was decreased by both jasmonates in a dose-dependent fashion. Once again, JA was found to be the more potent inhibitor of this activity of rAKR1C3, reaching significance when used at 400  $\mu$ mol/L ( $P = 0.025$ ).

To assess the effects of jasmonates on cellular AKR1C3 activity, KG1a cells were treated with either 1 mmol/L JA, 1 mmol/L MeJ, or ethanol (solvent control) overnight before incubation with [<sup>3</sup>H]PGD<sub>2</sub>. KG1a cells are derived from a patient with AML and display reasonable levels of PGD<sub>2</sub> 11-keto reductase activity (10). Interestingly, in these cellular assays, MeJ displayed a significantly greater inhibition of AKR1C3 than JA (Fig. 2B and C). These findings are in contrast to the *in vitro* inhibition of rAKR1C3 described above.

**JA and MeJ display differential cellular uptake.** Although the compounds are very similar, MeJ is a more lipophilic compound than JA. We therefore considered the possibility that the enhanced cellular potency of MeJ compared with JA to inhibit AKR1C3 may

be due to better accessibility into cells. We further hypothesized that the ester group of MeJ may be a target of cellular esterases, converting it to JA. To test these two hypotheses, HL-60 cells, an all-*trans* retinoic acid-sensitive cell line derived from a patient with AML (37), and KG1a cells, a cell line derived from a patient with AML that comprises highly undifferentiated blasts (38, 39), were exposed to jasmonates and cells were extracted to assess both uptake and metabolism using HPLC (Fig. 3). Interestingly, we did not find any evidence to suggest that MeJ was converted to JA intracellularly (Fig. 3A; data not shown). However, significantly more MeJ was recovered from cell extracts than JA ( $P = 0.006$ ; Fig. 3A and B). These data indicate that the greater potency of MeJ in cells is indeed associated with increased accessibility.

We proceeded to investigate whether the actions of jasmonates against AML cells are similar to those observed in response to other AKR inhibitors and if jasmonate effects on AML cell lines mirror those reported in other cancer cells. Furthermore, we wanted to assess whether MeJ is more potent than JA.

**Jasmonates reduce cell viability of myeloid leukemia cell lines.** Both JA and MeJ reduced cellular viability in a dose-dependent fashion, with MeJ being much more potent than JA. By day 5, there were very few, if any, viable cells remaining in doses >1 mmol/L MeJ, whereas at the same time point viable cells could readily be detected even at the highest dose of JA tested (4 mmol/L; Fig. 4A). These findings are in accordance with those of Fingrut and colleagues (25) using cell lines derived from T lymphoblastic lymphoma, melanoma, pancreatic carcinoma, and breast carcinoma. Together with our above observations, these data suggest that jasmonates mediate their antiproliferative actions via intracellular activities.

**Jasmonates induce increased levels of ROS in myeloid leukemia cells.** ROS, including hydrogen peroxide, hydroxyl radicals, and superoxide anions, are produced in cells as a consequence of electron leakage from the electron transport chain and can have detrimental effects on cells, causing lipid peroxidation, DNA adduct formation, and protein oxidation (40). ROS levels are significantly elevated in cancer cells when compared with their nontransformed counterparts (41). This has been proposed to represent an "Achilles' heel" and increasing ROS levels in tumor cells may present a valid goal for cancer therapy (42). Importantly, Oh and colleagues (30) showed that treatment of glioma cells with jasmonates leads to elevated ROS levels. We therefore, investigated whether jasmonates induce the production of ROS in myeloid leukemic cells. Interestingly, 24-hour exposure to either MeJ or JA

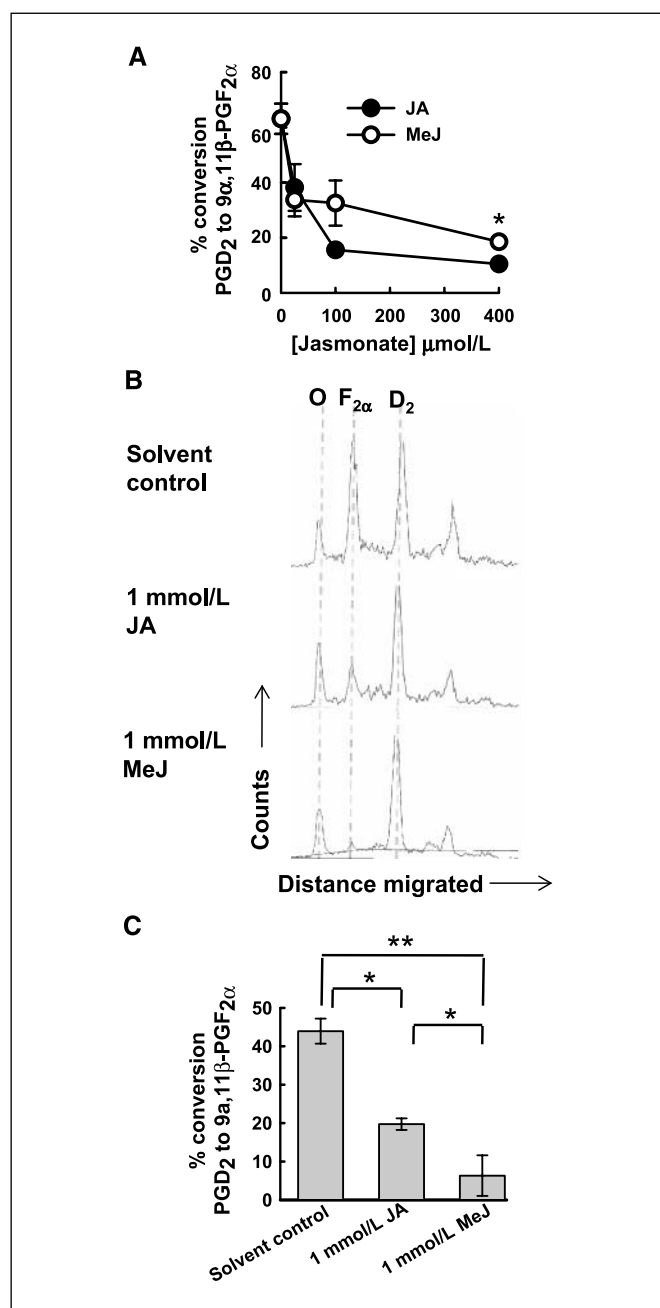
**Table 1.** All four AKR1C isomers are inhibited by jasmonates

	JA	MeJ
AKR1C1	106 $\pm$ 18	162 $\pm$ 37
AKR1C2	15 $\pm$ 3	49 $\pm$ 13
AKR1C3	21 $\pm$ 4	153 $\pm$ 28
AKR1C4	18 $\pm$ 3	ND

NOTE: Shown are calculated  $K_i$ s ( $\mu$ mol/L) for inhibition of reduction of phenanthrenequinone by rAKR1C1, rAKR1C2, rAKR1C3, and rAKR1C4 enzymes ( $n = 3$ ;  $\pm$ SE).

Abbreviation: ND, could not be determined.

<sup>2</sup> N.J. Davies, unpublished observations.

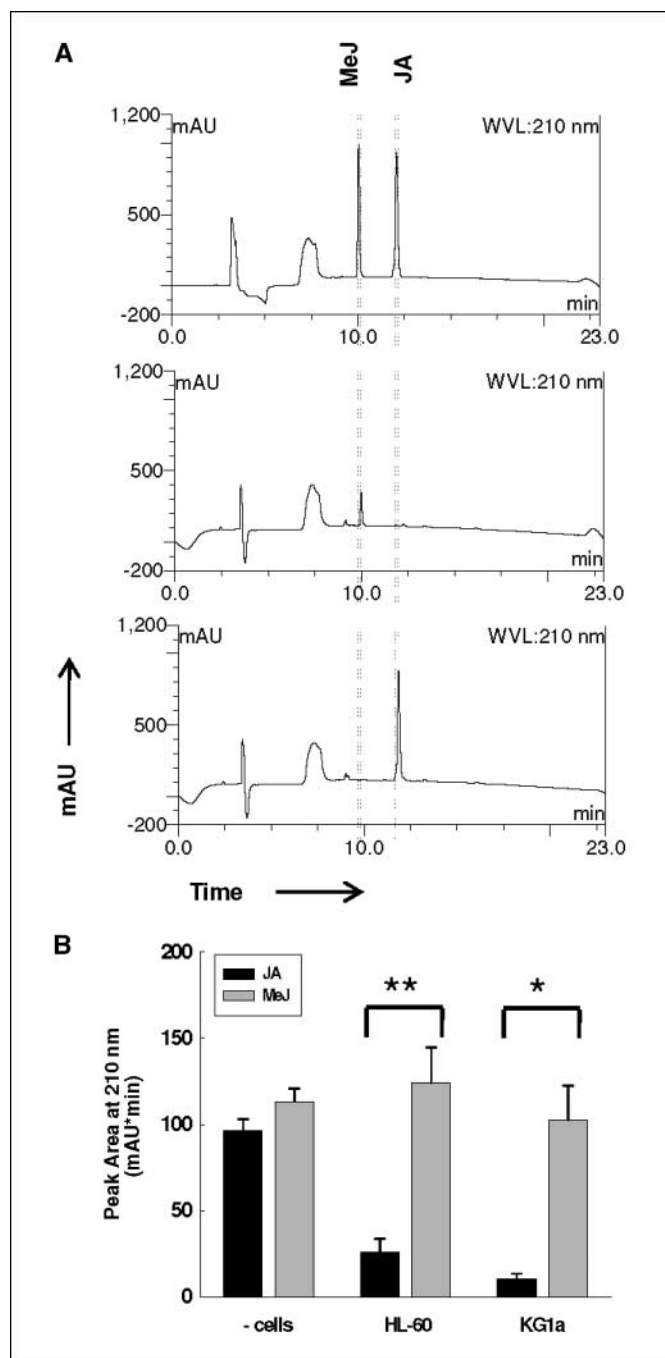


**Figure 2.** Jasmonates inhibit PGD<sub>2</sub> reduction by AKR1C3. **A**, average percentage conversion of PGD<sub>2</sub> to 9α,11β-PGF<sub>2α</sub> by rAKR1C3 in the presence of a dose titration of JA and MeJ. **B**, representative TLC traces showing the conversion of PGD<sub>2</sub> to 9α,11β-PGF<sub>2α</sub> by KG1a cells in the presence of no jasmonate (solvent control; *top*), 1 mmol/L JA (*middle*), and 1 mmol/L MeJ (*bottom*). Origin (O), 9α,11β-PGF<sub>2α</sub> peak (F<sub>2α</sub>), and PGD<sub>2</sub> peak (D<sub>2</sub>) are indicated. **C**, average percentage conversion of PGD<sub>2</sub> to 9α,11β-PGF<sub>2α</sub> by KG1a cells when cultured in the presence of either 1 mmol/L JA, 1 mmol/L MeJ, or solvent control (all graphs show *n* = 3). Bars, SE. \*, *P* < 0.05; \*\*, *P* < 0.01.

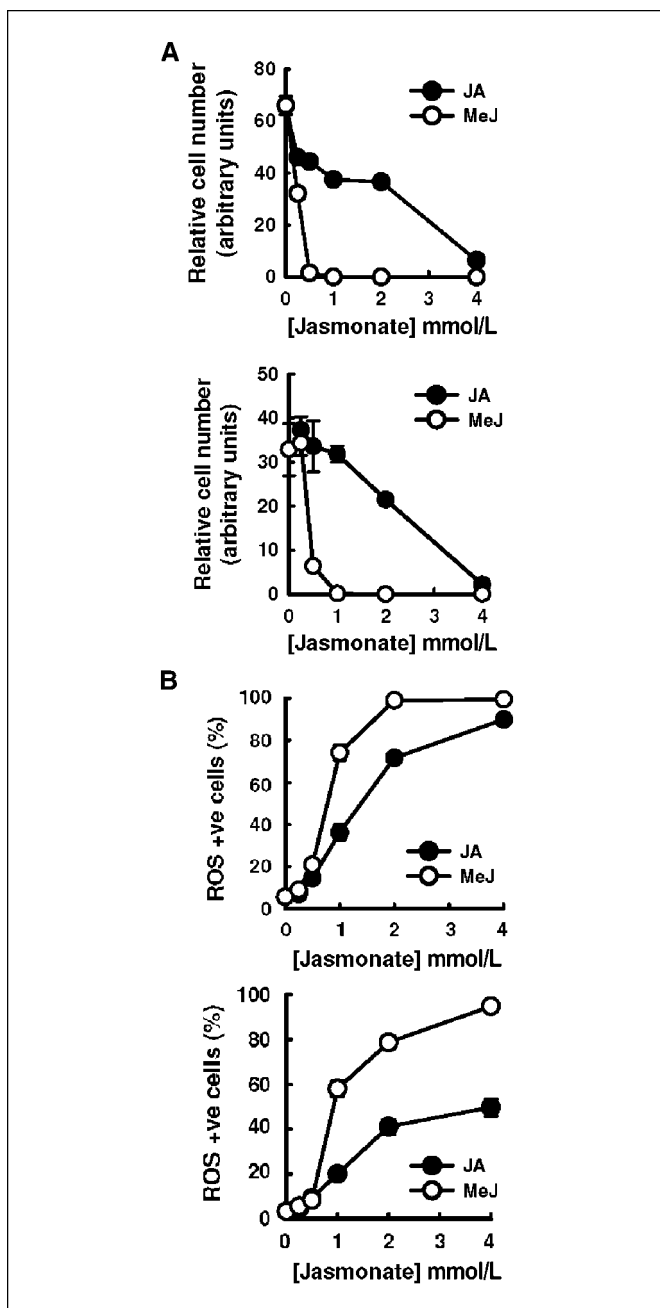
induced the dose-dependent formation of ROS in both HL-60 and KG1a cells (Fig. 4B). Although the effects of JA on ROS formation were again less pronounced than MeJ, they were notably more marked than the effects of JA on cell killing (Fig. 4). The data therefore indicate that the greater potency of MeJ to induce myeloid leukemic cell killing is not likely to be primarily due to its ability to induce elevated levels of ROS simply because JA also

generated significant levels of ROS without promoting cell death in these AML cells.

**MeJ affects mitochondrial activity.** Previously, Rotem and colleagues (29) showed that jasmonates induce mitochondriotoxic effects in mitochondria isolated from liver carcinoma and



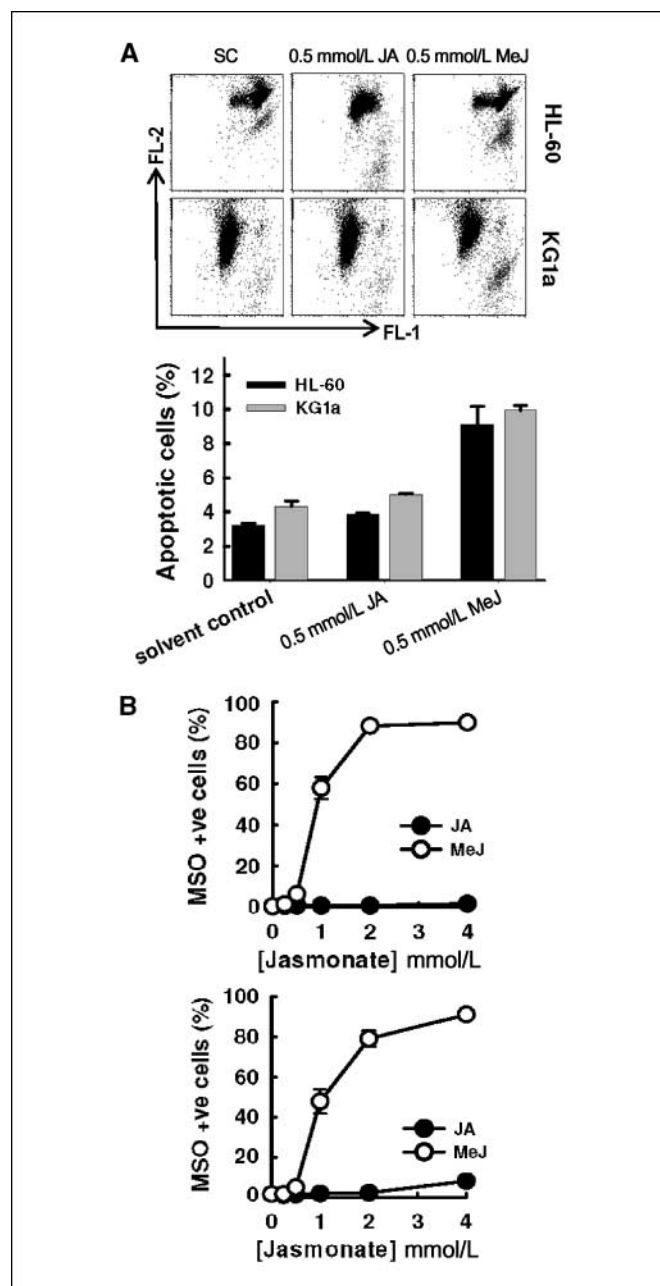
**Figure 3.** JA shows poor cellular uptake when compared with MeJ. Jasmonates were extracted either directly or intracellularly following 15-min incubation with HL-60 cells. Jasmonates were then separated by HPLC using a water + 0.1% TFA:acetonitrile + 0.1% TFA gradient and measured at an absorbance of 210 nm. **A**, representative HPLC traces of extractions of a mix of equal amounts of JA and MeJ without prior cell incubation (*top*) or intracellular extracts of JA (*middle*) or MeJ (*bottom*). **B**, intracellular (KG1a or HL-60) or uncultured (- cells) jasmonate extracts were separated using HPLC and peak areas were calculated by Chromleon software from the HPLC traces (all graphs show *n* = 3/4). Bars, SE. \*, *P* < 0.05; \*\*, *P* < 0.005.



**Figure 4.** Jasmonates induce ROS formation and reduce cellularity of myeloid leukemia cell lines. HL-60 and KG1a cells were cultured for 24 h and 5 d in the presence of increasing concentrations of JA and MeJ before analysis of ROS and cellularity respectively. *A*, cellularity was assessed using the CellTiter-Blue Cell Viability Assay in HL-60 (*top*) and KG1a (*bottom*) cells. Results were adjusted for feeding. *B*, levels of ROS were assessed by flow cytometry using carboxy-H<sub>2</sub>DCFDA in HL-60 (*top*) and KG1a (*bottom*) cells ( $n = 3$ ). Bars, SE.

T lymphoblastic leukemia cell lines and primary chronic lymphoblastic leukemia cells. These included mitochondrial membrane depolarization, osmotic swelling, and the release of cytochrome *c*. We therefore measured mitochondrial depolarization in jasmonate-treated HL-60 and KG1a cells using flow cytometry. Using the fluorochrome JC-1, polar mitochondria give high fluorescence detectable in the FL-2 channel and low fluorescence in the FL-1 channel. On depolarization, this is reversed and a high FL-1 and

low FL-2 signal is observed. As shown in Fig. 5A, 0.5 mmol/L MeJ significantly increased the proportion of HL-60 and KG1a cells displaying high FL-1/low FL-2 fluorescence indicative of mitochondrial depolarization ( $P = 0.0057$  for HL-60 and  $P = 0.0002$  for KG1a cells). Again, the potency of MeJ in this assay was greater than that of JA, which caused little or no increase in mitochondrial membrane depolarization at the doses tested. To further analyze



**Figure 5.** Jasmonates adversely affect mitochondria. The cell lines HL-60 and KG1a were cultured for 24 h and 5 d in the presence of increasing concentrations of JA and MeJ before analysis of MSO production and mitochondrial membrane depolarization, respectively. *A*, *top*, mitochondrial membrane depolarization was assessed by flow cytometry using JC-1. Representative fluorescence-activated cell sorting plots are shown for HL-60 (*top*) and KG1a (*bottom*) following treatment with ethanol (SC; left), 0.5 mmol/L JA (middle), or 0.5 mmol/L MeJ (right). *Bottom*, mean percentage of apoptotic cells. *B*, MSO levels were assessed by flow cytometry using MitoSOX Red in both HL-60 (*top*) and KG1a (*bottom*) cells (all graphs show  $n = 3$ ). Bars, SE.

the mitochondrial toxicity of jasmonates in intact cells, we next measured the generation of MSO, a by-product of the electron transport chain, forming as the result of inappropriate reaction between oxygen and electrons that have leaked from the respiratory chain.

Treatment of both HL-60 and KG1a cells with MeJ led to dose-dependent increases in MSO formation (Fig. 5B). However, there were very few cells that were positive for the production of MSO when treated with JA. Notably, there seemed to be an inverse correlation between the dose responses for MeJ-induced MSO generation and cell survival responses shown in Figs. 4A and 5B. Thus, the superior cell killing effects of MeJ over JA seem to be associated with the targeting of mitochondria and the generation of MSO rather than generic ROS generation. However, it remains to be determined whether the MeJ-induced MSO occurs as a direct effect of MeJ treatment or if it is in fact an epiphenomenon due to mitochondrial damage.

## Discussion

Increasingly, natural products from plants are being identified as possessing antitumor activities. Among many classes of molecule, these include plant hormones such as cytokinins (24) and auxins (23). Included in this list are members of the jasmonate family. There is a growing body of work investigating the antitumor effects of jasmonates both *in vitro* and *in vivo* (25, 26). Of particular interest here is the potential of jasmonates or derivatives thereof in hematolymphoid malignancy. The earliest reports of jasmonate activities included suppressed proliferation and induced apoptosis of AML, lymphoblastic leukemia, and lymphoma cell lines (25, 43, 44). This activity was shown to be selective because normal peripheral blood lymphocytes were spared even in mixed populations with chronic lymphocytic leukemia cells (29). Importantly, MeJ was shown to have *in vivo* activity in mice bearing EL-4 lymphoma xenografts (44).

Previous studies have implicated jasmonates in rapidly and selectively depleting cellular ATP in cancer cells by suppressing mitochondrial oxidative phosphorylation with the resultant generation of ROS. However, we observed ROS generation in response to both MeJ and JA and this did not correlate with the far more potent cyto-reductive activities of MeJ compared with JA. Instead, the potent actions of MeJ were found to strongly correlate with the specific generation of MSO. It therefore appears that it is more specifically the generation of MSO rather than overall ROS that triggers differentiation and apoptosis. Previously, the Flescher lab has shown that jasmonates directly target mitochondria (29, 45, 46). It therefore remains to be determined whether the MSO generation reported here is a direct effect of MeJ or is an epiphenomenon of mitochondrial damage. Furthermore, Ishii and colleagues (43) previously showed that MeJ-promoted HL-60 cell

differentiation was associated with increased mitogen-activated protein kinase (MAPK) activity. Treatment of HL-60 cells with the MAPK inhibitor PD98059 both diminished this activation and the differentiation seen in response to MeJ. This is of interest because activation of MAPK has recently been identified as a downstream effect of jasmonates in plants (47). Furthermore, a link has been suggested by Traore and colleagues (48) between increased mitochondrial-derived ROS and MAPK activation during myeloid differentiation. Taken together, these studies may suggest a link between MeJ-induced MSO generation and the subsequent activation of MAPK.

We and others have identified AKRs of the human AKR1C family as potential novel targets in leukemias, lymphomas, and solid tumors. Here, we have shown for the first time that jasmonates act as inhibitors of these enzymes. Importantly, although JA was the more potent inhibitor of rAKR1C proteins, MeJ jasmonate was better able to enter cells and accordingly was a better inhibitor of cellular AKR1C3 activity. The ability to inhibit AKRs adds to a growing list of potential mediators of the anticancer activities of jasmonates.

Besides studying the direct antitumor activities of Jasmonates, it is important to consider their exploitation as adjunctive therapy. In this regard, it is worth noting that AKR1C family members have been implicated in the inactivation of established chemotherapeutics, including daunorubicin, doxorubicin, and oracin (6, 49). Heyfets and Flescher (28) showed that administration of doxorubicin alongside MeJ had additive cytotoxicity compared with either agent alone against murine BCL1 leukemic cells both *in vitro* and *in vivo*. It is possible that this cooperativity displayed may be due, at least in part, to inhibition of the reduction of doxorubicin to the less toxic doxorubicinol by AKRs.

In conclusion, we have shown that AKR1C family members represent novel cellular targets for MeJ in both *in vitro* and cellular assays. It is this activity allied to the observations by others that MeJ can activate the MAPK pathway, as well as deplete cellular ATP levels, particularly in cancer cells that make this compound an exciting and a potentially important starting point for drug development.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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