Invest New Drugs (2012) 30:1878–1886
DOI 10.1007/s10637-011-9749-7

PRECLINICAL STUDIES

Aza-derivatives of resveratrol are potent macrophage migration inhibitory factor inhibitors

Yoshihiko Fujita · Rafiqul Islam · Kazuko Sakai · Hiroyasu Kaneda · Kanae Kudo · Daisuke Tamura · Keiichi Aomatsu · Tomoyuki Nagai · Hidekazu Kimura · Kazuko Matsumoto · Marco A. de Velasco · Tokuzo Arao · Tadashi Okawara · Kazuto Nishio

Received: 28 July 2011 / Accepted: 31 August 2011 / Published online: 13 September 2011

Summary Resveratrol (3, 4′, 5-trihydroxy-trans-stilbene), a natural phytoalexin found in grapes and wine, has anti-proliferative activity on human-derived cancer cells. In our study, we used a conventional condensation reaction between aldehydes and amines to provide a number of aza-resveratrol (3, 4′, 5-trihydroxy-trans- aza-stilbene) derivatives in an attempt to screen for compounds with resveratrol's action but with increased potency. Aza-resveratrol and its hydroxylated derivative (3, 4, 4′, 5-tetrahydroxy-trans- aza-stilbene) showed a more enhanced anti-proliferative effect than resveratrol in an MCF-7 breast carcinoma cell line. To identify the cellular targets of the aza derivatives of resveratrol, we conjugated the latter aza-stilbene compound with epoxy-activated agarose and performed affinity purification. Macrophage migration inhibitory factor (MIF), a proinflammatory cytokine, was identified as a major target protein in MCF-7 cell lysates using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS). The aza-resveratrol and its hydroxylated derivative, but not resveratrol, were also found to be potent inhibitors of MIF tautomerase activity, which may be associated with their inhibitory effects on MIF bioactivity for cell growth.

Keywords Resveratrol · Cancer · Macrophage migration inhibitory factor (MIF) · Tautomerase, CD74

Introduction

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine/growth factor that is known to contribute to inflammatory and autoimmune diseases. MIF is expressed under physiological conditions in a wide variety of tissues and cell types and is secreted as a consequence of the systemic stress response [1, 2]. Moreover, MIF has recently been implicated in multiple aspects of tumor growth including the control of cell proliferation and the promotion of angiogenesis [3, 4]. High levels of MIF expression have been found in human tumors including melanomas, breast carcinomas, prostate cancer, and adenocarcinoma of the lung [5, 6]. Secretion of MIF by tumor cells has been proposed to enhance tumor cell proliferation by autocrine amplification, as observed with other growth factors expressed by cancer cells [7–10]. Recent studies have shown that MIF signal transduction is initiated by binding to the transmembrane protein, CD74 [11, 12]. Inhibition of MIF-CD74 binding has been shown to attenuate tumor growth and angiogenesis [3]. It has also been suggested that, in addition to its extracellular role, MIF may have additional regulatory functions within the cell [13]. In this context, MIF has been shown to physically bind to p53 intracellularly, thereby protecting tumor cells from apoptosis [14]. MIF possesses the unusual ability to catalyze the tautomerization of the nonphysiological substrates D- and L-dopachrome methyl ester into their corresponding indole

Electronic supplementary material The online version of this article (doi:10.1007/s10637-011-9749-7) contains supplementary material, which is available to authorized users.

Y. Fujita · K. Sakai · H. Kaneda · K. Kudo · D. Tamura · K. Aomatsu · T. Nagai · H. Kimura · K. Matsumoto · M. A. de Velasco · T. Arao · K. Nishio (✉)
Department of Genome Biology,
Kinki University School of Medicine,
Osaka-Sayama,
589-8511, Osaka, Japan
e-mail: knishio@med.kindai.ac.jp

R. Islam · T. Okawara
Institute of Health Science, Kumamoto Health Science University,
Kumamoto 861-5598, Japan

derivatives, although the physiological role of the catalytic activity of MIF remains unknown [15]. Several small molecule tautomerase inhibitors of MIF have been reported [16–21]; some of these inhibitors—including the best characterized one, ISO-1[S,R-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester] [22, 23], inhibited the cytokine/growth factor activity of MIF at least marginally [24]. Recently, covalent inhibitors such as 4-iodo-6 phenylpyrimidine (4-IPP) and phenethyl isothiocyanate have been reported to effectively deactivate MIF by structural modification [25, 26]. Since covalent inhibitors are often not optimal as lead candidates, drug-like molecules that bind reversibly to MIF have also been sought.

Resveratrol is a polyphenolic compound that is found in grape skin and is classified as a phytoalexin, a class of antibiotics of plant origin [27–29]. This compound has been shown to have a wide spectrum of biological activity [30] including growth-inhibitory activity in several human cancer cell lines of diverse origin and in animal models of carcinogenesis [31–33]. However, its activity at a molecular level is not fully understood. The identification of the major molecular targets of resveratrol should provide an insight into the mechanisms of action. The main problem with resveratrol is that this molecule exerts an effect at high micromolar concentrations [34]; therefore, identifying its cellular targets is difficult.

Several types of resveratrol analogues have been synthesized to study the relation between structure and function [35–37]. However, preparation of analogues that retain the intramolecular stilbenic C=C double bond requires several steps and is therefore time-consuming. Pagliai et al. used the concept of click chemistry for the rapid synthesis of a series of resveratrol analogues and screened for compounds with an anti-proliferative effect using a panel of cell lines [38]. In the present study, we synthesized a series of resveratrol analogues using a conventional condensation reaction between aldehydes and amines. Two aza-resveratrol derivatives thus produced showed anti-proliferative activities that were more potent than that of their parent compound, resveratrol. We performed affinity purification using agarose beads conjugated with one of the resveratrol derivatives to isolate the molecular target of the compound(s) from cell lysates and identified an affinity-captured 12.5-kDa polypeptide as MIF. We demonstrated that the two aza-resveratrol derivatives, but not the parental resveratrol, bind to MIF and antagonize MIF binding to its cellular receptor, CD74, which most likely attenuate the cytokine/growth factor activity of MIF for cell proliferation. Such inhibitors may lead to the development of drugs for the treatment of cancer and, possibly, inflammatory and autoimmune diseases.

Materials and methods

Materials

Biochemical and molecular biology grade chemicals and reagents were purchased from various commercial vendors, including epoxy-activated agarose resin (12 atom linker, 33 μmol of epoxy group/mL of packed gel) and resveratrol (compound **1**), both of which were purchased from Sigma Chemical (St. Louis, MO).

Synthesis of aza-resveratrol derivatives

(*E*)-5{(4-hydroxyphenylimino)methyl}benzene-1,3-diol (Aza-resveratrol) (compound **2**) was prepared as follows. To a solution of triethylamine (101 mg, 1.0 mmol) in ethanol (15 mL), 4-Aminophenol hydrochloride (145.6 mg, 1.0 mmol) was added and the mixture was stirred at room temperature for 5 min. Next, 3,5-dihydroxybenzaldehyde (138 mg, 1 mmol) and acetic acid (1 drop) were added. The resulting solution was refluxed for 8 h. The solvent was then removed under reduced pressure and cold water (4 mL) was added to the residue. After 3–4 min, the solid mass was separated and collected by filtration followed by washing with water (3 mL) to produce compound **2** as an off-white powder; the yield was 167 mg (73%). The product was then recrystallized from a mixture of ethanol-toluene. The melting point was 223°C (decomposed).

^{1}H-NMR (DMSO-d_6): δ 6.31 (1H, s, CH), 6.76 (4H, s, CH), 7.14 (2H, s, CH), 8.38 (1H, s, CH), 9.43 (3H, br s, OH).

(*E*)-5{(4-hydroxyphenylimino)methyl}benzene-1,2,3-triol (compound **3**) was prepared as follows. To a solution of triethylamine (101 mg, 1.0 mmol) in ethanol (15 mL), 4-Aminophenol hydrochloride (145.6 mg, 1.0 mmol) was added and the mixture was stirred at room temperature for 5 min. Next, 3,4,5-trihydroxybenzaldehyde monohydrate (172 mg, 1 mmol) and acetic acid (1 drop) were added. The resulting solution was refluxed overnight. The solvent was then removed under reduced pressure and cold water (4 mL) was added to the residue. After 3–4 min, the solid mass was separated and collected by filtration followed by washing with water (3 mL) to produce compound **3** as an orange powder; the yield was 172 mg (70%). The product was then recrystallized from a mixture of ethanol-toluene. The melting point was 234°C.

^{1}H-NMR (DMSO-d_6): δ 6.75 (2H, d, J= 8.1 Hz, CH), 6.86 (2H, s, CH), 7.11 (2H, d, J= 8.4 Hz, CH), 8.27 (1H, s, CH), 8.73 (1H, br s, OH), 9.16 (2H, s, OH), 9.41 (1H, s, OH).

Cell lines and cultures

The DLD-1 colon carcinoma, MCF-7 breast carcinoma, A375 melanoma, MiaPaCa-2 pancreatic carcinoma, T24

bladder carcinoma, and A549 non-small cell lung carcinoma cell lines were cultured in DMEM medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), and the cell lines were maintained in a 5% CO_2-humidified atmosphere at 37°C.

MTT cell proliferation assay

Drug cytotoxicity was assessed in vitro using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In 96-well plates, 2×10^3 cells per well were plated in 200 μL of culture medium containing increasing drug concentrations. After 72 h of culture, 20 μL of MTT (5 mg/mL in PBS, obtained from Sigma) were added to each well and the plates were incubated for 2 h. The resulting formazan product was dissolved with DMSO, and the absorbance at a wavelength of 490 nm (A490) was read using a SPECTRAmax Microscope Spectrophotometer (Molecular Devices, Sunnyvale, CA).

Preparation of immobilized affinity column for compound 3

Immobilized affinity beads were prepared according to Wang et al. [39]. Briefly, epoxy-activated agarose (0.33 g) was suspended in 1 mL of ice-cold water for 5 min and washed extensively; then, 4.9 mg of compound **3** (dissolved in 0.5 mL of 0.1-M NaOH) was added, followed by overnight incubation at room temperature to allow for the chemical coupling of compound **3** to the agarose beads. To stop the reaction, 1 mL of 1-M sodium acetate buffer (pH5.0) containing 1 mM dithiothreitol (DTT) was added to the mixture to neutralize the unreacted epoxy groups. The compound **3**-immobilized beads were washed successively with 0.1-M sodium acetate (pH5.0) containing 1 mM of DTT and a series of 70%, 30%, 10%, and 0% graded ethanol. Controls consisted of mock-treated beads (prepared using an identical procedure except that no ligand was added).

Identification of intracellular targets of compound 3

MCF-7 cells were collected and lysed in a buffer containing 10 mM HEPES (pH7.5), 90 mM KCl, 1.5 mM $Mg(OAc)_2$, 1 mM DTT, 0.5% NP-40, 5% glycerol, 0.5 mM phenylmethylsulphonyl fluoride and Complete™ protease inhibitors (Roche Diagnostics, Basel, Switzerland). Cell-free extracts were obtained by centrifugation for 10 min in a refrigerated microcentrifuge. Fifty microliters of compound **3**-immobilized affinity beads (**3**AB) and mock-treated beads (MB) were incubated with 1 mL of cell lysate containing 3.0 – 5.0 mg of protein for 16 h at 4°C with constant rotation. After incubation, the beads were thoroughly washed with lysis buffer. The bound protein was eluted by boiling the beads in the presence of SDS reducing buffer (62.5 mM Tris–HCl [pH6.8], 10% glycerol, 2% SDS, 0.025% bromophenol blue, and 700 mM β-mercaptoethanol). The beads were pelleted, and the resulting supernatant was resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The total protein was silver-stained. The lowest band corresponding to an apparent molecular mass of 12.5 kDa was excised from the gel and digested, and the resulting peptide fragments were analyzed using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS) (Voyager-DE STR;Applied Biosystems, Foster City, CA).

Immunoblot analysis

The immunoblot analysis for MIF protein detection was performed essentially as described previously [40] using anti-MIF antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunofluorescence staining

To detect the cell surface expression of CD74, cells grown to subconfluence on cover slips were treated with a medium containing monoclonal anti-CD74 antibody (Santa Cruz Biotechnology) at a concentration of 5 μg/mL and incubated for 10–20 min at 37°C. After washing three times with the medium, the cells were incubated at 37°C for another 10–20 min in a medium containing 10 μg/mL of Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR). After washing three times with HBSS (pH7.3), the cells were fixed in 4% formaldehyde for 30 min at room temperature (RT), rinsed in PBS, and mounted on slide glasses using Vectashield (Vector Laboratories, Burlingame, CA). The cells were observed using a confocal microscope (LSM5 Pascal; Zeiss, Oberkochen, Germany).

Expression and purification of recombinant MIF (rMIF)

The MIF cDNA was generated by PCR using the primers 5′ – CCGGGCATGCCGATGTTCATCGTAAACAC – 3′ and 5′ – GGGAGATCTGGCGAAGGTGGAGTTGTTC – 3′ and excised with Sph I and Bgl II. The resulting cDNA fragment was cloned into the Sph I and Bgl II site of an expression vector, pQE-70 (Qiagen, Hilden, Germany). His-tagged MIF was expressed in *E. coli* XL-1 blue and purified using a metal affinity column (Talon; Invitrogen) according to the manufacturer's instructions. About 180 mg of purified MIF was obtained from 1 l of culture.

Dopachrome tautomerase assay

L-dopachrome methyl ester was prepared at 2.4 mM through the oxidation of L-3,4-dihydroxyophenylalanine

methyl ester (4.8 mM) with an equi-volume of sodium periodate (16 mM). The activity was determined at room temperature by adding dopachrome methyl ester (60 μL) to a 96-well plate containing 50 nM of rMIF in 140 μL of 50 mM potassium phosphate buffer (pH6.0) with 0.5 mM EDTA; the decrease in absorbance at 475 nm was then measured for 5 min. The compounds intended for the testing of the inhibitory effect were dissolved in DMSO and added to the plate with MIF prior to the addition of the dopachrome.

MIF – CD74 binding analysis

A375 cells were seeded in a 96-well plate (~8,000 cells/well) and cultured overnight. The cells were then incubated with 80 μM of FITC-rMIF conjugates in 100 μL of HBSS (pH7.3) in the presence or absence of rMIF (4 μM), compound **2** (50 μM), compound **3** (10 and 50 μM) and ISO-1 (50 μM) and observed using a fluorescence microscope (IX 71; Olympus, Japan). The conjugation of MIF to FITC was performed using the SureLINK^{TM} FITC Labeling Kit (Socochim, Lausanne, Switzerland) according to the manufacturer's protocol.

Results

Chemistry

An attempt was made to develop a reaction that enabled the quick preparation of a number of resveratrol analogues for studies of their structures and functions. We took advantage of a conventional condensation reaction using aldehydes and amines to quickly obtain a variety of aza-derivatives of resveratrol. Figure 1 shows resveratrol (compound **1**) and the two analogues (compounds **2** and **3**) that were synthesized and used in this study.

Anti-proliferative effect of aza-compounds

To evaluate the effect of resveratrol and its aza-derivatives on the cell viability of several cancer cell lines, an MTT assay was performed (Fig. 2). Aza-resveratrol (compound **2**) inhibited the growth of MCF-7 to a greater degree than resveratrol (compound **1**) (Fig. 2a). The IC_{50} value at 72 h posttreatment with compound **1** was more than 100 μM, whereas it was only 28 μM after treatment with compound **2**. This difference suggests that the conversion from a stilbene structure (with C=C) to an aza derivative (with C=N) enhanced the anti-proliferation effect in this cell line. The addition of the hydroxyl group at position 4 of compound **2** (compound **3**) further increased the anti-proliferation effect on MCF-7 (Fig. 2a). Similar sensitivity to each compound was observed by MiaPaCa-2 pancreatic cancer cells and to a lesser extent T24 bladder carcinoma and A375 melanoma cells, whereas DLD-1 colon cancer and A549 lung cancer cells were considerably more resistant to all compounds (Fig. 2b). To explore the molecular mechanism by which the aza-derivatives of resveratrol exerted their anti-proliferative activities (against a subset of the cancer cell lines), we attempted to identify the cellular targets of aza-compound **3**.

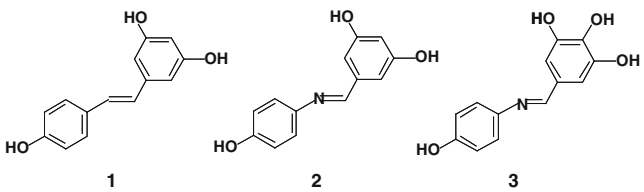
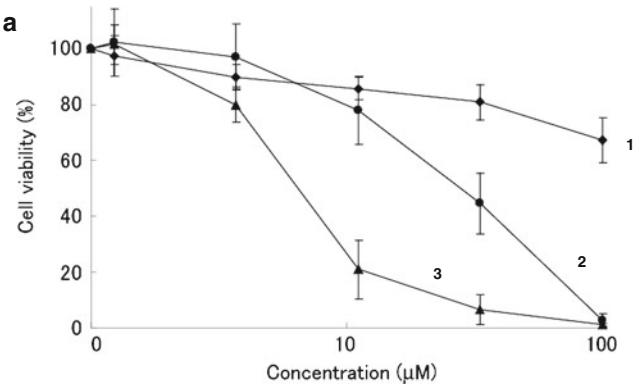


Fig. 1 Structures of resveratrol (1) and its aza-derivative compounds 2 and 3



b

Cell line	Origin	IC_{50} (μmol/L)		
		1	2	3
MCF7	Breast Cancer	>100	28.2	6.4
MiaPaca-2	Pancreatic Cancer	99.8	47.1	8.3
T24	Bladder Cancer	>100	65.9	24.3
A375	Melanoma	>100	40.8	27.2
DLD-1	Colon Cancer	94.4	73.2	65.4
A549	Lung Cancer	86.2	75.9	62.1

Fig. 2 Antiproliferative activity of resveratrol (1) and its aza-derivative compounds 2 and 3. **a** Growth inhibition of MCF-7 cells after treatment with resveratrol (compound **1**) and its derivatives, (compounds **2** and **3**), for 72 h. Exponentially dividing cells were treated with 0, 1.23, 3.7, 11.1, 33.3, or 100 μM of the compounds. Cell viability was determined using the MTT assay. The percentage of growth was calculated, with 100% representing control cells treated with 0.1% DMSO alone. The results are the mean±SDs from three separate experiments. **b** IC_{50}s of compounds **1**, **2** and **3** for proliferation of several cells calculated using the MTT assay as done in **a**. Numbers represent average IC_{50} from two independent experiments

Identification of high-affinity binding protein molecules of aza-compounds

Polyphenols are advantageous in that they are able to couple with agarose beads even without any molecular modification, such as aminoalkylation. We simply reacted compound **3** with epoxy-activated agarose beads for immobilization. The chemical coupling of the resveratrol analogue occurred at one or more of the 4 available hydroxyl groups (3, 4, 5, 4′), resulting in compound **3**-immobilized affinity beads (**3**AB). Lysates from the MCF-7 cells were then incubated with the **3**AB. Proteins that bound to 3AB were eluted, separated by SDS-PAGE and visualized using silver staining. A prominent band with an apparent molecular mass of 12.5 kDa showed a specific retention on **3**AB. This band could not be detected in mock-treated control beads (CB), indicating that the interaction was not caused by nonspecific binding to the agarose beads (Fig. 3a). The band was excised from the gel and analyzed using MALDI-TOF MS, resulting in the identification of the 12.5-kDa as macrophage migration inhibitory factor (MIF) (Fig. 3b). For confirmation, the eluates were resolved using SDS-PAGE and immunoblotted with an anti-MIF antibody. MIF was clearly shown to bind with the **3**AB and not with the mock-treated CB (Fig. 3c). Specific affinity between MIF and **3**AB was shown by the pretreatment of the lysate with compound **3**, which disrupted the MIF binding to the **3**AB. We also found that compound **2** (aza-resveratrol) inhibited the subsequent binding of MIF to the **3**AB. To our surprise, compound **1** (resveratrol) had no effect on their binding. These results led us to conclude that the slight structural difference between compound **1** (with C=C) and **2** (with C=N) was responsible for their affinity with MIF.

Tautomerase activity

MIF possesses the ability to catalyze the tautomerization of DL-dopachrome methyl esters into their corresponding indole derivatives [15]. If both aza-compounds **2** and **3** bind to MIF, they may share a function with small molecules so far identified as tautomerase inhibitors of MIF [16–19]. We tested if resveratrol (compound **1**) and its aza-derivatives (compounds **2** and **3**) could inhibit the enzymatic activity of MIF (Fig. 4). The two aza-compounds inhibited the tautomerase activity of rMIF in a dose-dependent manner with IC_{50} values of 18.7 and 8.9 μM for compounds **2** and **3**, respectively. However, resveratrol showed no inhibitory effect (Fig. 4). These results suggest that antagonists of MIF's enzymatic reaction may be correlated with the elevated inhibitory potential of these compounds in MCF-7 cells.

MIF-CD74 binding assay

Compounds **3** (or **2**) were capable of exerting an anti-proliferative effect by targeting MIF and possibly inhibiting the binding of MIF to its cell surface receptor, CD74. We examined this possibility by monitoring cell surface binding of MIF-CD74 using rMIF labeled with FITC (FITC-rMIF), which would enable us to stain CD74-expressing cells fluorescently. We first used MCF-7 cells to detect rMIF-CD74 binding, but sufficient fluorescence intensity was not obtained**,** possibly because the detectable level of CD74 in this cell line was too low for this purpose. We performed immunofluorescence analysis to screen for an appropriate cell line (Fig. 5a)**.** The A375 melanoma cell line was thus chosen as a cell line to be monitored for

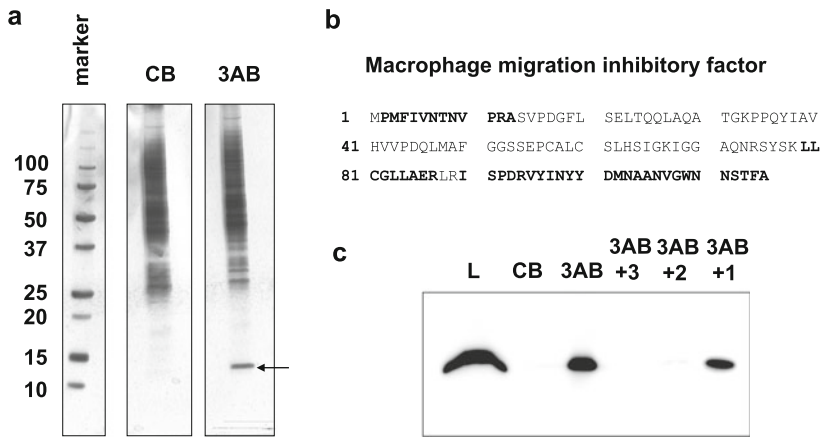


Fig. 3 Molecular identification and characterization of the affinity-captured protein. **a** Affinity beads immobilized with compound **3** (**3**AB) or control beads (CB) were incubated with a MCF-7 cell lysate. Protein bound to the columns was eluted and resolved using SDS-PAGE. The highlighted protein band of 12.5-kDa was excised from the gel and identified using MALDI-TOF MS as the cytokine MIF. **b** Matched peptides identified using MALDI-TOF MS were shown in bold. **c** Immunoblot detection of MIF binding to **3**AB. MCF-7 cell lysate (lane 1), an eluate recovered from CB (lane 2), an eluate from **3**AB (lane 3), an eluate from **3**AB when the lysate was preincubated for 30 min with 50 μM of compound **3** (lane 4), compound **2** (lane 5) or compound **1** (lane 6) is shown

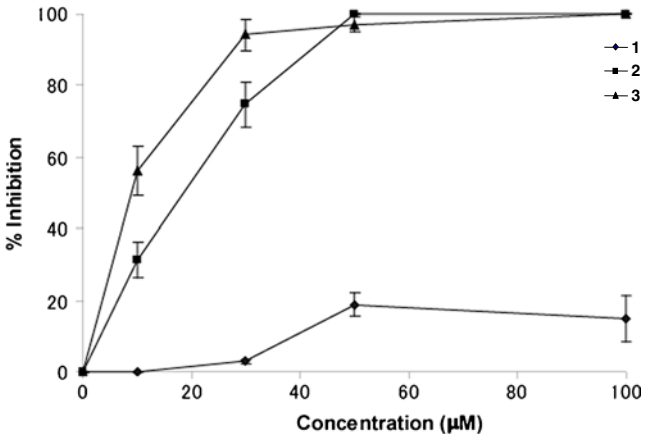


Fig. 4 Two aza compounds inhibit dopachrome tautomerase activity. The indicated concentrations of resveratrol (compound **1**) and its aza-derivatives (compounds **2** and **3**) were preincubated with 50 nM rMIF in 50 mM potassium phosphate buffer followed by the addition of L-dopachrome methyl ester at a final concentration of 0.72 mM. Spectrophotometric measurements were made at λ=475 nm by monitoring the rate of decolorization of L-dopachrome in comparison to a standard curve

FITC-rMIF binding activity, as it expressed the largest amount of cell surface CD74 of all the cell lines that were analyzed. In contrast to the receptor (CD74), abundant ligand protein (MIF) was uniformly expressed in all these cell lines (Suppl. Fig. 1), as has been observed in many cancer types (5, 6). As expected, FITC-MIF fluorescently stained A375 cells. Preincubation of the A375 cells with excess unlabeled rMIF inhibited the binding of FITC-MIF, indicating the specific binding of MIF to its cell surface receptor (Fig. 5B 1 and 2). Aza-compound **3** at 50 μM, but not at 5 μM, inhibited the binding. Compound **2** at 50 μM also inhibited the binding, but Iso-1 at 50 μM had no effect on MIF-CD74 binding (Fig. 5B 3–6).

Discussion

One informative approach to investigating the mechanisms of the diverse functions of resveratrol is to identify its molecular targets. We started with the synthesis of resveratrol analogues and screening of these compounds for the ability to inhibit cell growth at a lower IC_{50} value than that of resveratrol. Such compounds with increased activities are likely to be more strongly associated with resveratrol's molecular targets. To prepare resveratrol analogues, we used a conventional condensation reaction between aldehydes and amines. This reaction is suitable for combinatorial chemistry and has the great advantage of enabling the quick preparation of a number of aza-derivatives of resveratrol. As a preliminary study, we prepared aza-resveratrol **2**, in which the C=C double bond of resveratrol was replaced by C=N, together with its 4′-hydroxylated derivative **3**, and compared their anti-proliferative effects with that of resveratrol **1** on MCF-7, a breast cancer cell line that has frequently been used to study the effect of resveratrol [41–46]. Both aza-resveratrol derivatives (**2** and **3**) inhibited cell proliferation more potently than

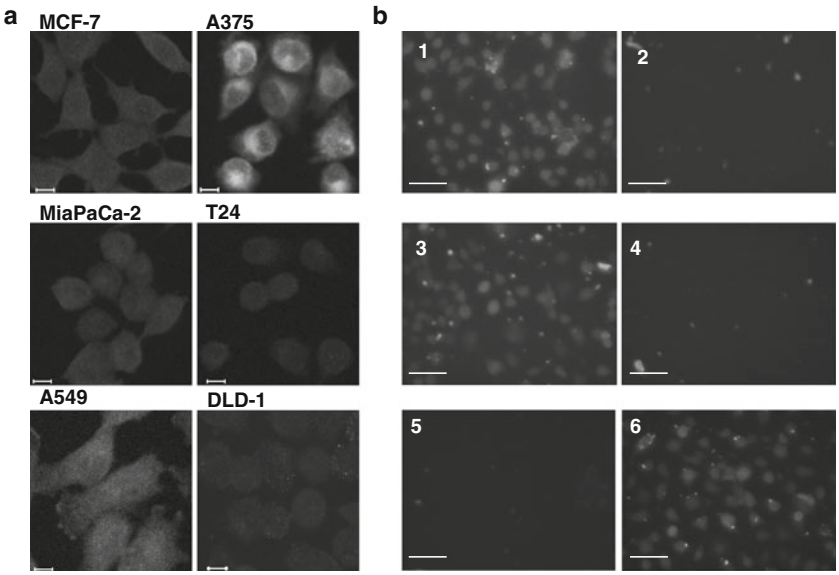


Fig. 5 Fluorescence analysis of FITC-MIF binding to CD74-expressing cells. **a** Immunofluorescence analysis of cell surface expression of CD74 for MCF-7, A375, MiaPaCa-2, T24, A549 and DLD-1 cells. Cells were treated with5 μg/mL of monoclonal anti-CD74 antibody and incubated for 10–20 min at 37°C. After washing three times with HBSS (pH 7.3), the cells were treated with 10 μg/mL of Alexa Fluor 488 goat anti-mouse IgG, which were then fixed in 4% formaldehyde. *Scale bar*, 10 μm. **b** Fluorescence microscopy images of A375 cells pretreated with (1) none, (2) rMIF (50 μg/mL), (3) compound **3** (5 μM), (4) compound **3** (50 μM), (5) compound **2** (50 μM) and (6) ISO-1 (50 μM) prior to the addition of FITC-MIF (2 μg/mL) are shown. *Scale bar*, 100 μm

compound **1**, with derivative **3** exhibiting the most potent IC_{50} value of less than 10 μM. Hydroxylation at specific positions of resveratrol has been demonstrated to show enhanced radical scavenging activity, resulting in the growth inhibition of HL-60 leukemic cells due to the formation of antioxidant-derived prooxidants [47]. More polyhydroxylated aza-resveratrol derivatives need to be synthesized to clarify how the antioxidative properties of these compounds contribute to the anti-proliferation effect on cancer cells.

When conjugated to epoxy-activated agarose beads, derivative **3** permitted the identification of MIF as the most prominent cellular binding protein of the aza-resveratrol derivatives. We initially expected MIF to be a target of resveratrol itself as well. However, the preincubation of cell lysates with resveratrol **1** allowed the ability of cellular MIF to bind to the affinity beads to be retained, while preincubation with compounds **2** or **3** abolished its binding to the beads, suggesting that MIF could only interact with the aza-resveratrol derivatives (Fig. 3c). Wang et al. recently isolated a resveratrol-targeting protein using immobilized resveratrol affinity beads and identified this protein as dihydronicotinamide riboside quinone reductase 2 (NQO2) [39]. This protein was not detected using our compound **3**-immobilized affinity beads (data not shown), which again explains the different biochemical properties between resveratrol and its aza-derivatives. *In silico* modeling of resveratrol **1** and the two aza-resveratrol derivatives **2** and **3** at the tautomerase active site of MIF revealed no obvious conformational changes arising from a change in only one atom (C to N) at the double bond moiety of resveratrol. Some interaction may occur between the protein and the N atom of aza-resveratrol to lead this compound specifically into the binding pocket of MIF. In accordance with this finding, compounds **2** and **3,** but not **1,** exerted an inhibitory effect on the tautomerase activity of MIF (Fig. 4).

Several small-molecule tautomerase inhibitors of MIF have been identified so far, and these molecules have shown some inhibitory effects on MIF-dependent cell migration and glucocorticoid regulation [1, 48], although none have inhibited cell proliferation as effectively as compound **3**. MIF signal transduction is initiated by the binding of MIF to the transmembrane protein CD74 [11, 12]. Accordingly, a small molecule would not inhibit the biological activity of MIF with regard to cell growth unless it was capable of impairing MIF-CD74 binding, even if it functions as a tautomerase inhibitor of MIF. Recently, Cournia et al. performed a virtual screening by docking compounds from a virtual library into the MIF tautomerase active site, and among the compounds with positive results, several compounds that also inhibit MIF-CD74 binding were experimentally identified. According to their elaborate MIF-CD74 binding assay, the IC_{50} values of some of these compounds were below 5 μM (24). Iso-1 ((S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester), a well-characterized MIF tautomerase inhibitor, was only marginally active in MIF-CD74 binding. They demonstrated that most MIF-CD74 binding antagonists provide some tautomerase inhibition and ascribed this action to the notion that the interaction of MIF with CD74 occurs in the vicinity of the tautomerase active site [24]. Because 50 μM of our aza-polyphenols **2** and **3** inhibited the binding of fluorescently labeled rMIF to CD74 expressed on the cell surface, they appeared to have already gained the MIF-CD74 binding antagonism that causes their anti-proliferation effect on MCF-7 cells. Notably, Iso-1 at 50 μM failed to block MIF binding to CD74 in our system (Fig. 5).

The expression level of CD74 may not be necessary for compounds **2** and **3** to exert their anti-proliferation effect**,** since A375 cells (which have a higher level of CD74 expression on their cell surfaces than MCF-7) are less susceptible to the anti-proliferation effect of compounds **2** and **3** (Figs. 2 and 5). The molecular mechanism by which the unequal sensitivity to these MIF inhibitors occur among the cell lines remains to be elucidated (Fig. 2b).

In summary, we have identified a unique family of small compound tautomerase inhibitors of MIF that act as antagonists of MIF-CD74 binding to inhibit MIF-dependent tumor cell growth. We propose that mono-hydroxylated aza-resveratrol (compound **3)** represents a unique class of anti-MIF therapeutic agents, although further studies are needed to elucidate the antitumor efficacy of this novel lead compound.

Acknowledgements We thank Eiko Honda for performing the MALDI-TOF MASS spectrometry, Yoshitaka Horiuchi for the microscopic analysis and Tomoko Kitayama for cell experiments.

Disclosure of potential conflicts of interest No potential conflicts of interests were disclosed.

Funding This work was supported by funds for the Comprehensive 3rd term of the 10-Year Strategy for Cancer Control, a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (19209018).

References

1. Calandra T, Bernhagen J, Metz CN, Spiegel LA, Bacher M, Donnelly T, Cerami A, Bucala R (1995) MIF as a glucocorticoid-induced modulator of cytokine production. Nature 377:68–71
2. Bernhagen J, Calandra T, Mitchell RA, Martin SB, Tracey KJ, Voelter W, Manogue KR, Cerami A, Bucala R (1993) MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. Nature 365:756–759
3. Meyer-Siegler KL, Iczkowski KA, Leng L, Bucala R, Vera PL (2006) Inhibition of macrophage migration inhibitory factor or its

receptor (CD74) attenuates growth and invasion of DU-145 prostate cancer cells. J Immunol 177:8730–8739
4. Hagemann T, Robinson SC, Thompson RG, Charles K, Kulbe H, Balkwill FR (2007) Ovarian cancer cell-derived migration inhibitory factor enhances tumor growth, progression, and angiogenesis. Mol Cancer Ther 6:1993–2002
5. Meyer-Siegler K (2000) Increased stability of macrophage migration inhibitory factor (MIF) in DU-145 prostate cancer cells. J Interferon Cytokine Res 20:769–778
6. del Vecchio MT, Tripodi SA, Arcuri F, Pergola L, Hako L, Vatti R, Cintorino M (2000) Macrophage migration inhibitory factor in prostatic adenocarcinoma: correlation with tumor grading and combination endocrine treatment-related changes. Prostate 45:51–57
7. Hudson JD, Shoaibi MA, Maestro R, Carnero A, Hannon GJ, Beach DH (1999) A proinflammatory cytokine inhibits p53 tumor suppressor activity. J Exp Med 190:1375–1382
8. Fingerle-Rowson G, Petrenko O, Metz CN, Forsthuber TG, Mitchell R, Huss R, Moll U, Muller W, Bucala R (2003) The p53-dependent effects of macrophage migration inhibitory factor revealed by gene targeting. Proc Natl Acad Sci USA 100:9354–9359
9. Mitchell RA, Liao H, Chesney J, Fingerle-Rowson G, Baugh J, David J, Bucala R (2002) Macrophage migration inhibitory factor (MIF) sustains macrophage proinflammatory function by inhibiting p53: regulatory role in the innate immune response. Proc Natl Acad Sci USA 99:345–350
10. Nguyen MT, Lue H, Kleemann R, Thiele M, Tolle G, Finkelmeier D, Wagner E, Braun A, Bernhagen J (2003) The cytokine macrophage migration inhibitory factor reduces pro-oxidative stress-induced apoptosis. J Immunol 170:3337–3347
11. Leng L, Metz CN, Fang Y, Xu J, Donnelly S, Baugh J, Delohery T, Chen Y, Mitchell RA, Bucala R (2003) MIF signal transduction initiated by binding to CD74. J Exp Med 197:1467–1476
12. Shi X, Leng L, Wang T, Wang W, Du X, Li J, McDonald C, Chen Z, Murphy JW, Lolis E, Noble P, Knudson W, Bucala R (2006) CD44 is the signaling component of the macrophage migration inhibitory factor-CD74 receptor complex. Immunity 25:595–606
13. Nguyen MT, Beck J, Lue H, Funfzig H, Kleemann R, Koolwijk P, Kapurniotu A, Bernhagen J (2003) A 16-residue peptide fragment of macrophage migration inhibitory factor, MIF-(50–65), exhibits redox activity and has MIF-like biological functions. J Biol Chem 278:33654–33671
14. Jung H, Seong HA, Ha H (2008) Critical role of cysteine residue 81 of macrophage migration inhibitory factor (MIF) in MIF-induced inhibition of p53 activity. J Biol Chem 283:20383–20396
15. Lubetsky JB, Dios A, Han J, Aljabari B, Ruzsicska B, Mitchell R, Lolis E, Al-Abed Y (2002) The tautomerase active site of macrophage migration inhibitory factor is a potential target for discovery of novel anti-inflammatory agents. J Biol Chem 277:24976–24982
16. Senter PD, Al-Abed Y, Metz CN, Benigni F, Mitchell RA, Chesney J, Han J, Gartner CG, Nelson SD, Todaro GJ, Bucala R (2002) Inhibition of macrophage migration inhibitory factor (MIF) tautomerase and biological activities by acetaminophen metabolites. Proc Natl Acad Sci USA 99:144–149
17. Orita M, Yamamoto S, Katayama N, Aoki M, Takayama K, Yamagiwa Y, Seki N, Suzuki H, Kurihara H, Sakashita H, Takeuchi M, Fujita S, Yamada T, Tanaka A (2001) Coumarin and chromen-4-one analogues as tautomerase inhibitors of macrophage migration inhibitory factor: discovery and X-ray crystallography. J Med Chem 44:540–547
18. Zhang X, Bucala R (1999) Inhibition of macrophage migration inhibitory factor (MIF) tautomerase activity by dopachrome analogs. Bioorg Med Chem Lett 9:3193–3198
19. Dios A, Mitchell RA, Aljabari B, Lubetsky J, O'Connor K, Liao H, Senter PD, Manogue KR, Lolis E, Metz C, Bucala R, Callaway DJ, Al-Abed Y (2002) Inhibition of MIF bioactivity by rational design of pharmacological inhibitors of MIF tautomerase activity. J Med Chem 45:2410–2416
20. Crichlow GV, Cheng KF, Dabideen D, Ochani M, Aljabari B, Pavlov VA, Miller EJ, Lolis E, Al-Abed Y (2007) Alternative chemical modifications reverse the binding orientation of a pharmacophore scaffold in the active site of macrophage migration inhibitory factor. J Biol Chem 282:23089–23095
21. Dabideen DR, Cheng KF, Aljabari B, Miller EJ, Pavlov VA, Al-Abed Y (2007) Phenolic hydrazones are potent inhibitors of macrophage migration inhibitory factor proinflammatory activity and survival improving agents in sepsis. J Med Chem 5:1993–1997
22. Al-Abed Y, Dabideen D, Aljabari B, Valster A, Messmer D, Ochani M, Tanovic M, Ochani K, Bacher M, Nicoletti F, Metz C, Pavlov VA, Miller EJ, Tracey KJ (2005) ISO-1 binding to the tautomerase active site of MIF inhibits its pro-inflammatory activity and increases survival in severe sepsis. J Biol Chem 280:36541–36544
23. Cheng KF, Al-Abed Y (2006) Critical modifications of the ISO-1 scaffold improve its potent inhibition of macrophage migration inhibitory factor (MIF) tautomerase activity. Bioorg Med Chem Lett 16:3376–3379
24. Cournia Z, Leng L, Gandavadi S, Du X, Bucala R, Jorgensen WL (2009) Discovery of human macrophage migration inhibitory factor (MIF)-CD74 antagonists via virtual screening. J Med Chem 52:416–424
25. Winner M, Meier J, Zierow S, Rendon BE, Crichlow GV, Riggs R, Bucala R, Leng L, Smith N, Lolis E, Trent JO, Mitchell RA (2008) A novel, macrophage migration inhibitory factor suicide substrate inhibits motility and growth of lung cancer cells. Cancer Res 68:7253–7257
26. Brown KK, Blaikie FH, Smith RA, Tyndall JD, Lue H, Bernhagen J, Winterbourn CC, Hampton MB (2009) Direct modification of the proinflammatory cytokine macrophage migration inhibitory factor by dietary isothiocyanates. J Biol Chem 284:32425–32433
27. Langcake P, Pryce RJ (1977) A new class of phytoalexins from grapevines. Experientia 33:151–152
28. Hain R, Bieseler B, Kindl H, Schroder G, Stocker R (1990) Expression of a stilbene synthase gene in Nicotiana tabacum results in synthesis of the phytoalexin resveratrol. Plant Mol Biol 15:325–335
29. Soleas GJ, Diamandis EP, Goldberg DM (1997) Resveratrol: a molecule whose time has come? And gone? Clin Biochem 30:91–113
30. Goldberg DM, Tsang E, Karumanchiri A, Diamandis E, Soleas G, Ng E (1996) Method to assay the concentrations of phenolic constituents of biological interest in wines. Anal Chem 68:1688–1694
31. Mahyar-Roemer M, Katsen A, Mestres P, Roemer K (2001) Resveratrol induces colon tumor cell apoptosis independently of p53 and precede by epithelial differentiation, mitochondrial proliferation and membrane potential collapse. Int J Cancer 94:615–622
32. Tinhofer I, Bernhard D, Senfter M, Anether G, Loeffler M, Kroemer G, Kofler R, Csordas A, Greil R (2001) Resveratrol, a tumor-suppressive compound from grapes, induces apoptosis via a novel mitochondrial pathway controlled by Bcl-2. Faseb J 15:1613–1615
33. Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 275:218–220
34. Pervaiz S (2003) Resveratrol: from grapevines to mammalian biology. Faseb J 17:1975–1985

35. Stivala LA, Savio M, Carafoli F, Perucca P, Bianchi L, Maga G, Forti L, Pagnoni UM, Albini A, Prosperi E, Vannini V (2001) Specific structural determinants are responsible for the antioxidant activity and the cell cycle effects of resveratrol. J Biol Chem 276:22586–22594
36. Matsuoka A, Takeshita K, Furuta A, Ozaki M, Fukuhara K, Miyata N (2002) The 4′-hydroxy group is responsible for the in vitro cytogenetic activity of resveratrol. Mutat Res 521:29–35
37. Murias M, Handler N, Erker T, Pleban K, Ecker G, Saiko P, Szekeres T, Jager W (2004) Resveratrol analogues as selective cyclooxygenase-2 inhibitors: synthesis and structure-activity relationship. Bioorg Med Chem 12:5571–5578
38. Pagliai F, Pirali T, Del Grosso E, Di Brisco R, Tron GC, Sorba G, Genazzani AA (2006) Rapid synthesis of triazole-modified resveratrol analogues via click chemistry. J Med Chem 49:467–470
39. Wang Z, Hsieh TC, Zhang Z, Ma Y, Wu JM (2004) Identification and purification of resveratrol targeting proteins using immobilized resveratrol affinity chromatography. Biochem Biophys Res Commun 323:743–749
40. Tanaka K, Arao T, Maegawa M, Matsumoto K, Kaneda H, Kudo K, Fujita Y, Yokote H, Yanagihara K, Yamada Y, Okamoto I, Nakagawa K, Nishio K (2009) SRPX2 is overexpressed in gastric cancer and promotes cellular migration and adhesion. Int J Cancer 124:1072–1080
41. Pozo-Guisado E, Lorenzo-Benayas MJ, Fernandez-Salguero PM (2004) Resveratrol modulates the phosphoinositide 3-kinase pathway through an estrogen receptor alpha-dependent mechanism: relevance in cell proliferation. Int J Cancer 109:167–173
42. Li Y, Liu J, Liu X, Xing K, Wang Y, Li F, Yao L (2006) Resveratrol-induced cell inhibition of growth and apoptosis in MCF7 human breast cancer cells are associated with modulation of phosphorylated Akt and caspase-9. Appl Biochem Biotechnol 135:181–192
43. Pozo-Guisado E, Alvarez-Barrientos A, Mulero-Navarro S, Santiago-Josefat B, Fernandez-Salguero PM (2002) The antiproliferative activity of resveratrol results in apoptosis in MCF-7 but not in MDA-MB-231 human breast cancer cells: cell-specific alteration of the cell cycle. Biochem Pharmacol 64:1375–1386
44. Tang HY, Shih A, Cao HJ, Davis FB, Davis PJ, Lin HY (2006) Resveratrol-induced cyclooxygenase-2 facilitates p53-dependent apoptosis in human breast cancer cells. Mol Cancer Ther 5:2034–2042
45. Gehm BD, McAndrews JM, Chien PY, Jameson JL (1997) Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. Proc Natl Acad Sci USA 94:14138–14143
46. Joe AK, Liu H, Suzui M, Vural ME, Xiao D, Weinstein IB (2002) Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. Clin Cancer Res 8:893–903
47. Murias M, Jager W, Handler N, Erker T, Horvath Z, Szekeres T, Nohl H, Gille L (2005) Antioxidant, prooxidant and cytotoxic activity of hydroxylated resveratrol analogues: structure-activity relationship. Biochem Pharmacol 69:903–912
48. Leech M, Metz C, Bucala R, Morand EF (2000) Regulation of macrophage migration inhibitory factor by endogenous glucocorticoids in rat adjuvant-induced arthritis. Arthritis Rheum 43:827–833