Synthesis, Antitumor Evaluation, and Apoptosis-Inducing Activity of Hydroxylated (*E*)-Stilbenes

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The parallel solution-phase synthesis of a series of 30 monohydroxylated (*E*)-stilbene analogues is described. In vitro screening revealed low micromolar activity (GI_{50}) against the MDA MB 468 breast cancer cell line. Activity in MDA MB 468 cells correlated with the ability to induce apoptosis following drug treatment by the most potent agents in the series, e.g., **5dy** and **5jy**, an observation further reinforced by AnnexinV-FITC analysis and fluorescence microscopy.

Introduction

A number of hydroxylated stilbenes derived from natural sources with a range of interesting biological activities have been reported.[1] Most notable among these from the antitumor perspective are the trihydroxylated *trans*-stilbene resveratrol[2] (isolated from edible materials such as grape skins, peanuts, and red wine) and the hydroxylated *cis*-stilbene combretastatin A-4[3] (from the African bush willow *Combretum caffrum*), shown in Figure 1.

The intriguing cardioprotective and cancer chemopreventative activity associated with resveratrol has prompted several reports describing the synthesis and antitumor evaluation of resveratrol analogues. For example, Roberti and co-workers have described the synthesis and biological evaluation of resveratrol analogues as apoptosis-inducing agents.[4] Also, Kim and co-workers have reported the synthesis of a range of (protected) *trans*-stilbene analogues and their evaluation as human cytochrome P450 (CYP) inhibitors to find a potent and selective inhibitor of the isoform CYP1B1.[5]

Because of our ongoing interest in the antitumor evaluation of novel chemical structures arising from the chemical oxidation of bioactive phenols,[6] we became interested in the synthesis of novel monohydroxylated stilbenes structurally related to resveratrol. Our reasons for choosing monohydroxylated (rather than polyhydroxylated) stilbenes at this stage were twofold: first, to enable the rapid parallel synthesis of a range of substituted analogues for antitumor evaluation and, second, to simplify and control subsequent oxidation chemistry. In this paper we report the solution-phase parallel synthesis and antitumor evaluation of a library of 30 monohydroxylated *trans*-stilbenes containing a variety (H, OMe, and F) of ring substituents.

Chemistry

Interest in the biological properties of resveratrol has resulted in the development of a number of synthetic methods toward this trihydroxystilbene, most commonly using the Wittig reaction and its variants to install the ethylenic bridge. Unfortunately these methods often lead to low yields of trans product and/or low *E*/*Z* selectivity and produce triphenylphosphine oxide as a byproduct, necessitating chromatographic purification. Recently Guiso and co-workers have reported the synthesis of resveratrol, exclusively in the (*E*)-form in good yield, via Heck reaction between a protected styrene derivative and protected *p*-iodophenol.[7]

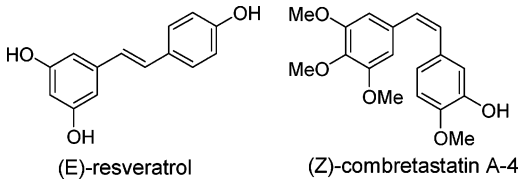

Figure 1. Structures of (*E*)-resveratrol and (*Z*)-combretastatin A-4.

We wished to develop our own strategy toward the solution-phase synthesis of (*E*)-stilbenes to produce new products in parallel without the need to resort to column chromatography and in quantities for full characterization and biological profiling (>50 mg). We chose to make use of Horner−Wadsworth−Emmons olefination chemistry, methodology possessing the key advantages of both (*E*)-specificity and easy removal of the water-soluble dialkylphosphoric acid byproduct.[8] A related strategy toward the synthesis of a substituted (*E*)-stilbene library for evaluation as human cytochrome P450 1B1 inhibitors was recently reported.[5]

The overall scheme for the parallel solution-phase synthesis of our hydroxylated (*E*)-stilbene library is shown in Scheme 1. Reaction of the (10) substituted benzylphosphonic acid diethyl esters (**1a**−**j**) under Horner−Emmons−Wadsworth conditions with 2-, 3-, or 4-methoxymethyl-substituted hydroxylbenzaldehydes (**2x**−**z**) afforded 30 novel protected monohydroxystilbene derivatives (**3ax**−**jz**) exclusively in the (*E*)-conformation in each case (no (*Z*)-isomer could be detected by ^{1}H NMR analysis). Among a range of protecting groups examined, the methoxymethyloxy (MOM) protecting group was found to be optimal, offering both easy attachment to and removal from the phenol and stability under Horner−Emmons−Wadsworth reaction conditions.

Parallel reactions could be carried out on either Carousel reaction systems mounted on conventional hotplate stirrers or on a Stem block format. A slight excess of benzaldehyde (**2x**−**z**) was required in each case to

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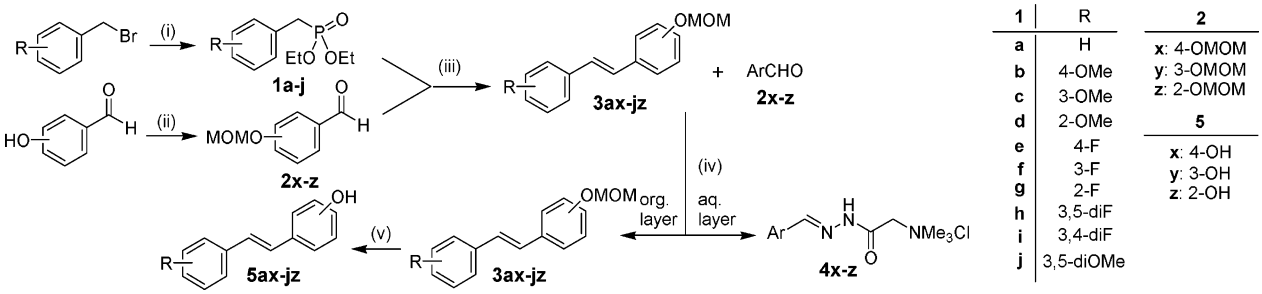
Scheme 1. Parallel Solution-Phase Synthesis of Hydroxylated (*E*)-Stilbenes[a]



[a] Reagents: (i) $P(OEt)_3$; (ii) CH_3OCH_2Cl, CH_2Cl_2; (iii) MeONa, DMF, 100 °C; (iv) Girard's reagent, AcOH, PPTS, MeOH; (v) PPTS, MeOH.

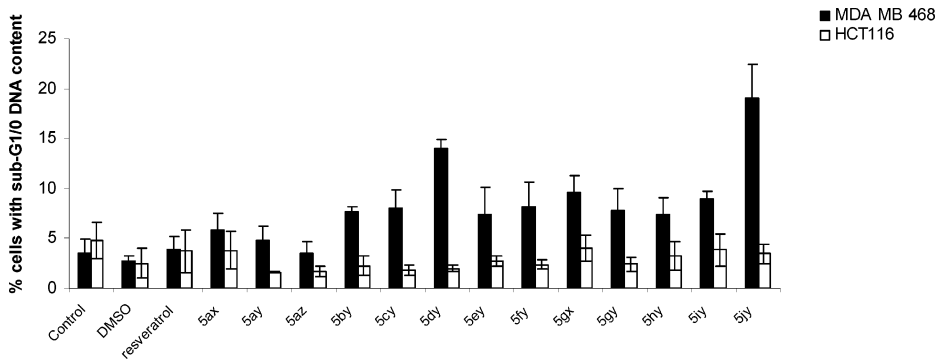


Figure 2. Flow cytometric analysis of cell cycle distribution in MDA MB 468 and HCT 116 cells treated with selected hydroxylated stilbenes (10 μM) for 48 h. Results are expressed as percent of cells with sub-G1/0 DNA content, indicative of potentially apoptotic cell populations. Results are the mean of three repeat experiments ± SEM.

ensure that reaction went to completion; excess benzaldehyde can conveniently be removed using Girard's reagent ((carboxymethyl)trimethylammonnium chloride hydrazide) and acetic acid, giving a readily removed water-soluble benzaldehyde hydrazide derivative (**4x**−**z**) by simple partition between organic and aqueous phases. Removal of the MOM-protecting group was carried out under mild conditions using pyridinium *p*-toluene sulfonate (PPTS) in methanol to afford 30 hydroxylated (*E*)-stilbenes (**5ax**−**jz**) in high overall yield and purity and *without necessitating chromatographic purification at any stage*.

Biological Results and Discussion

In Vitro Activity against Human Cancer Cell Lines. Evaluation of the growth-inhibitory properties of hydroxylated (*E*)-stilbenes was undertaken in two human cancer cell lines, MDA MB 468 (breast) and HCT 116 (colon), using the MTT assay following 72 h of drug exposure.[9] In all cases, the human mammary carcinoma cell line MDA MB 468 was the most sensitive cell line examined with GI_{50} values in the low micromolar range, with agents **5jy** (0.96 μM), **5iz** (1.1 μM), **5gy** (1.4 μM), and **5iy** (1.6 μM) being the most potent compounds (testing in triplicate; see Supporting Information for details). The relative sensitivity of 3-hydroxy-(*E*)-stilbenes (among directly comparable structures) toward MDA MB 468 (with the exception of 2-hydroxystilbene **5iz**) is noteworthy. The growth inhibitory potencies of hydroxylated (*E*)-stilbenes against the colon cancer cell line HCT 116 were found to be lower, in most cases giving GI_{50} values within the micromolar range (15 to >100 μM). Resveratrol itself was tested in the MDA MB 468 and HCT 116 cell lines but was found to be inactive (GI_{50} > 40 μM) in both cell lines.

Flow Cytometric Analysis. The effects of selected hydroxylated stilbenes on the cell cycle distribution of HCT116 and MDA MB 468 cells were studied by flow cytometric analysis of cellular DNA content.[10] A selection of hydroxylated stilbenes (plus controls) were chosen for cell cycle analysis on the basis of cell line potency in MDA MB 468 and HCT 116 cancer cell lines, comprising 3-hydroxystilbene derivatives **5ay**−**5jy**, unsubstituted hydroxystilbenes **5ax**−**5az** (R = H), the 4-hydroxystilbene **5gx** (R = 2-F), and resveratrol plus controls (no drug and DMSO only).

MDA MB 468 and HCT116 cells were treated with 10 μM of selected hydroxylated stilbenes for 48 h. Collected cells were stained using propidium iodide (PI), then the DNA content was analyzed by flow cytometry. Results are expressed as the percentage cell population in the sub-$G_{0/1}$ phase of the cell cycle and are shown in Figure 2. Cells with sub-$G_{0/1}$ DNA content are designated as potentially apoptotic.

Inspection of Figure 2 reveals that following 48 h of treatment with test agents (**5ax**−**5jy**) there was a significantly higher population of cells in sub-G1/0 in MDA MB 468 cells compared to HCT 116. This observation of higher apoptotic potential in MDA MB 468 cells concurs with the superior growth inhibitory activity of hydroxylated stilbenes in MDA MB 468 cells. Also noteworthy is the observation that in all stilbenes examined, with the exception of the relatively insensitive **5az**, the percentage of apoptotic sub-G1/0 MDA MB 468 cells following drug treatment was higher than for resveratrol. Previous flow cytometric studies of DNA content of sensitive cancer cell lines (e.g., HL-60 human promyelocytic leukaemia) have demonstrated an increase in pre-G1 population following treatment with resveratrol and related stilbenes.[11]

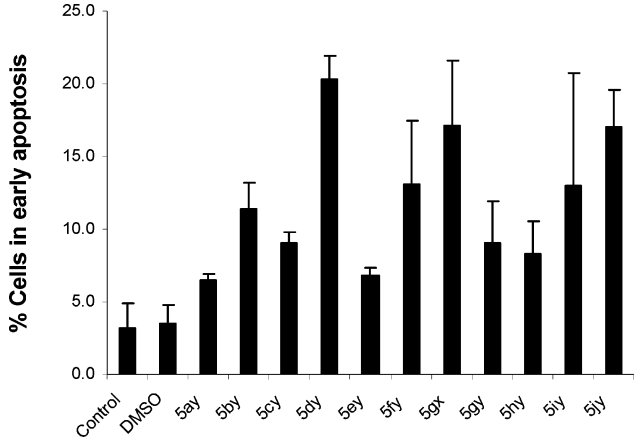


Figure 3. AnnexinV-PI analysis in MDA MB 468 cells, following 48 h of drug treatment at 10 μM, demonstrating early apoptotic events (an increase in AnnexinV-FITC binding) without concurrent increase in PI staining. Results are the mean of three repeats ± SEM.

AnnexinV-FITC/Propidium Iodide Analysis of Apoptosis. An early apoptotic event is the translocation of phosphatidylserine (PS) from the inner to the outer membrane leaflet of the cell. This can be detected using fluorescein-labeled annexinV (annexinV-FITC), a Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS. Combined with PI (used as an indicator of cell integrity), a measure of percentage cell population in early apoptosis can be achieved. Following treatment of MDA MB 468 cells according to the AnnexV-FITC detection kit protocol (Santa Cruz), cells were analyzed by flow cytometry. AnnexinV-FITC fluorescence was collected in FL1, and propidium iodide fluorescence was collected in FL3. Cells showing increased FL1 fluorescence without a concurrent increase in FL3 fluorescence are considered to be in early apoptosis. Where an increase is seen in both fluorescence channels, the cells are considered to be in late apoptosis or to have undergone necrosis.

The results of the AnnexinV-FITC/PI analysis for early apoptosis are shown in Figure 3. As for the flow cytometric analysis (Figure 2), a subset of hydroxylated stilbenes was chosen according to their growth inhibitory properties in the MDA MB 468 human cancer cell line. Figure 3 clearly indicates that the highest percentage of cells deemed to be in early apoptosis (increase in AnnexinV-FITC binding without concurrent increase in PI staining) was observed following treatment (10 μM, 48 h) with compounds **5dy**, **5jy**, and **5gx**. Once again, induction of apoptosis correlated with antiproliferative activity in MDA MB 468 cells, particularly for the relatively potent 3-hydroxylated stilbenes **5dy** and **5jy**. Notably, the percentage of cells deemed to be in early apoptosis at 10 μM for compound **5dy** (20%) was much higher than for resveratrol itself (4%) but much lower than for the standard anticancer agent camptothecin (37% at 250 nM); see Supporting Information for further details.

Fluorescence Microscopy. Further confirmation of the ability of the most potent hydroxylated stilbenes in the series to induce apoptosis was obtained by analysis of drug-treated MDA MB 468 cell populations (10 μM, 48 h) by fluorescence microscopy (Figure 4). Following drug treatment the cells were resuspended in acridine orange, spotted on glass slides, and visualized under a fluorescence microscope. Cells showing shrinkage accompanied by chromatin condensation and marginalization are defined as being apoptotic.

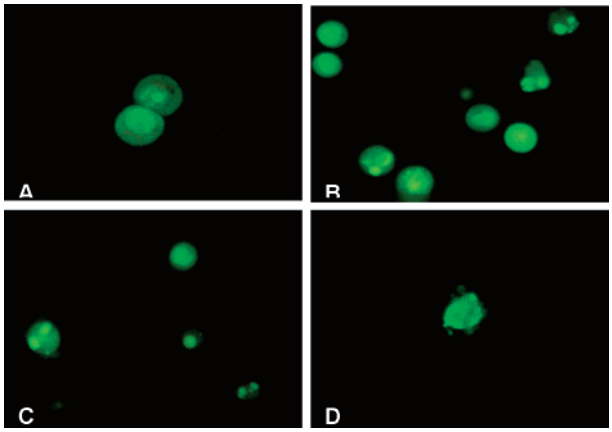


Figure 4. Fluorescence microscopy images showing appearance of apoptotic cells (MDA MB 468) following drug treatment at 10 μM for 48 h: (A) control; (B) **5by**; (C) **5dy**; (D) **5jy**.

Conclusions

In this study we have synthesized a novel family of monohydroxylated (*E*)-stilbenes and studied their ability to inhibit the growth and induce apoptosis in human tumor cell lines. The potential of these hydroxylated stilbenes as antitumor agents will probably not reside in the potency of their growth-inhibitory activity per se. Rather, the potential of these agents may manifest itself in their ability to induce apoptosis in a selective manner and inhibit a number of cancer relevant biological targets, as is the case for resveratrol. We have observed moderately potent growth-inhibitory effects most notably in the MDA MB 468 cell line, particularly for the 3-hydroxylated stilbene derivatives such as **5dy** and **5jy**. This agent also produced the highest percentage of sub-$G_{0/1}$ cells in MDA MB 468 cell lines following drug treatment, indicative of apoptotic potential, a finding reinforced by fluorescence microscopy of apoptotic cells. Our search for mechanistic targets underpinning the antitumor activity of these novel agents will be reported at a future date.

Experimental Section

General Method for Parallel Synthesis of Hydroxylated (*E*)-Stilbene MOM Ethers. Substituted phosphonic acid diethyl esters (**1a**−**j**) (10 mmol) were dissolved in dry DMF (10 mL). Sodium methoxide (20 mmol) and 18/6-crown ether (2 mmol) were added, and the mixture was stirred at room temperature for 5 min. Hydroxybenzaldehyde methoxymethyl ethers (**2x**−**z**) (15 mmol) dissolved in dry DMF (5 mL) were added dropwise at 0 °C, and the mixture was stirred at room temperature for 1 h followed by heating to 120 °C for 5 h. The reactions were quenched with water (20 mL), and the mixture was extracted with Et_2O (3 × 20 mL). The ether was evaporated in vacuo, and the residues were redissolved in dichloromethane (15 mL). Girard's reagent T (6 mmol) and acetic acid (60 mmol) were added, and the reaction stirred at room temperature for 2 h. After quenching with water (15 mL), the organic layers were collected, washed with brine (3 × 15 mL) and aqueous Na_2CO_3 (3 × 15 mL), dried over $MgSO_4$, and evaporated in vacuo to afford the substituted methoxymethyloxystyrylbenzenes (**3ax**−**3jz**) as oils.

General Method for Synthesis of Hydroxylated (*E*)-Stilbenes. The substituted hydroxylated (*E*)-stilbene meth-

oxymethyl ethers (**3ax**−**jz**) (5 mmol) were dissolved in methanol (40 mL). Pyridinium *p*-toluene sulfonate (50 mmol) was added, and the mixture was heated to reflux until completion of the reaction (monitored by TLC, eluant $CHCl_3$). The solvent was then evaporated in vacuo, and the residues were dissolved in brine (40 mL) and extracted with diethyl ether (3 × 25 mL). The organic layers were collected, washed with water (3 × 25 mL), dried over $MgSO_4$, and evaporated. Recrystallization from MeOH/water afforded the pure substituted hydroxylated (*E*)-stilbenes.

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Supporting Information Available: General methods for synthesis of benzylphosphonic acid diethyl esters and MOM-protected hydroxylbenzaldehydes, physical and spectroscopic data for compounds **3ax**−**3jz** and **5ax**−**5jz**, general biological protocols, a table of in vitro GI_{50} values in MDA MB 468 and HCT 116 cell lines, and annexinV-PI analysis in MDA MB 468 cells for camptothecin, resveratrol, and compound **5dy** (dose−response). This material is available free of charge via the Internet at http://pubs.acs.org.

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