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Cinnamaldehydes Inhibit Cyclin Dependent Kinase 4/Cyclin D1

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Abstract—A series of cinnamaldehydes was synthesized for the study of inhibitory activity against cyclin dependent kinases (CDKs). A couple of compounds selectively inhibited cyclin D1-CDK4 with an IC_{50} value of 7–18 μM.

In eukarytotes, cell cycle control is the major regulatory mechanism of cell growth.[1] In the past decade, enormous advances have been made in our understanding of the molecules that mediate the control of cell cycle.[2,3] Cyclins, cyclin dependent kinases (CDKs), CDK inhibitors (CDIs), retinoblastoma protein (pRB), and the E2F transcription factor family that form the central core of cell-cycle machinery have been identified and their encoding genes cloned. Among these are the cyclin dependent kinases, which phosphorylate a large variety of substrates directly involved in the cell cycle progression, such as pRb or lamins.[4] These kinases are activated by association with their specific regulatory cyclin proteins and by various post-translational modifications. Primary regulators of the progression are the D-type cyclins (D1, D2, D3), cyclin E, CDK2, and CDK4. CDK4 is activated early in G1 by interactions with D-type cyclins, whereas CDK2 is activated later in G1 by interactions with cyclin E. Pharmaceutical attention has been focused on the cyclin D1-CDK4 complexes, because the cumulative data show that the activity of the cyclin D1-CDK4 is critical for proliferation of many tumors. Therefore, selective inhibitors of cyclin D1-CDK4 will be useful for the development of anticancer drugs.[5]

In the course of a screening of herbal medicines for the development of new types of anticancer drugs, we found that 2′-hydroxycinnamaldehyde (**IIa**), isolated from the stem bark of *Cinnamonum cassia* Blume (Lauraceae), showed various in vitro activities on farnesyl transferase, angiogenesis, immunmodulation, cell–cell adhesion, and a cytotoxicity against tumor cell lines.[6–10] Compound **IIa** also strongly inhibited the growth of tumors without loss of body weight in nude mice.[9] However, it is very difficult to identify the antitumor mechanism of the compound. The antitumor activity of **IIa** is likely to derive from a combination of various independent and additive mechanisms. Therefore, compound **IIa** was measured to have an inhibition activity against CDKs, the major regulatory kinases in cell cycle and we found that **IIa** fairly inhibited cyclin D1-CDK4 with an IC_{50} value of 5 μg/mL (Table 1). We thus planned to synthesize a series of derivatives of **IIa** and measured the CDK inhibition activity of the cinnamaldehydes.

Based on the previous results, the propenal and free phenolic hydroxy groups of the cinnamaldehydes were identified as key functional groups. However, it was reported, when the propenal group was converted to propenol or propanal, that the antitumor activity of the compounds disappeared.[10] Therefore, we have focused on making a cinnamaldehyde library based upon templates **IIa** and **IIb**, and substitution of 2′- or 3′-OH in Scheme 1. In particular, **IIc** and **IId** were synthesized to introduce chloro and nitro groups into the 2′-OH position to compare the inhibition activity between 2′-OH and other substituents.

The compounds were synthesized using standard literature methods as outlined in Scheme 1. 2′ or 3′-Substituted cinnamic acids were converted to their corresponding esters, followed by reduction of the esters with diisobutyl aluminum hydride to give cinnamyl alcohols. Manganese(IV) oxide oxidation of these alcohols gave cinnamaldehydes (**II**). 2′ or 3′-Hydroxycinnamaldehydes (**IIa**

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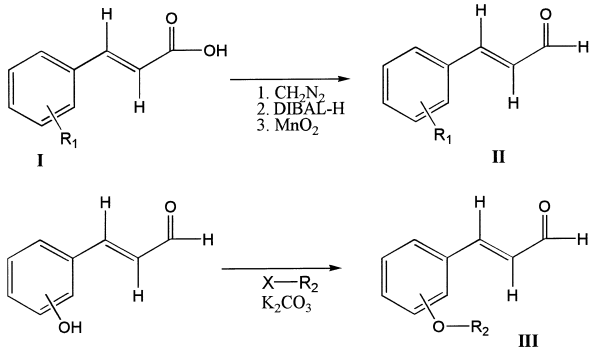
and **IIb**) were treated with a variety of substituted benzyl halides to provide compounds **III** in a good yield (80–90%).[10]

Structures of the synthesized compounds were identified by ^{1}H NMR and HRMS. Especially, the propenal group of the compounds was clearly identified by the observed ^{1}H NMR chemical shifts and coupling constants, for example of the aldehyde at δ 9.6 (d, 7.7 Hz), and propenyl moiety at δ 8.0 (d, 16.3 Hz) and δ 6.8 (dd, 7.8 and 16.3 Hz) for **IIa**. The methylene group of benzyl substituted compound **III** was also detected by ^{1}H NMR at δ 4.5–5.0.[11]

The enzyme inhibitory activity of the synthesized compounds was determined by measuring the phosphorylation of histone H1 for cyclin E-CDK2 and cyclin B-CDK1, and Rb protein for cyclin D1-CDK4, respectively.[12–14] Their inhibitory activities towards the enzymes are presented as percentages of the control condition in which the enzyme assay was carried out in the absence of inhibitors. The inhibitory activity of cinnamaldehydes against cyclin D1-CDK4 is summarized in Table 1 and olomoucine (IC_{50} 10 μM for cyclin E-CDK2)[12] and flavopiridol (IC_{50} 100 nM for cyclin D1-CDK4),[15,16] well known CDK2 and 4 inhibitors, respectively, were used as standard compounds. 3′-Substituted



Scheme 1.

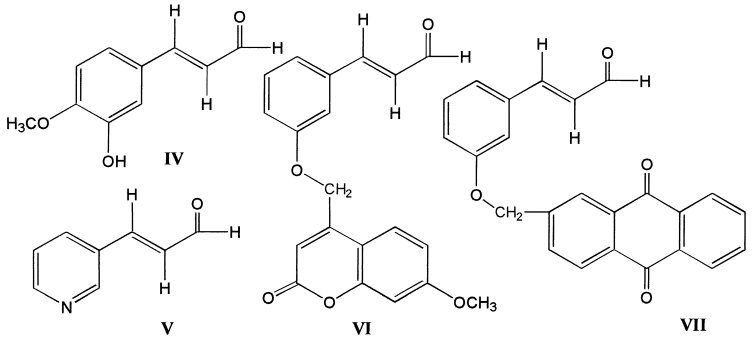


Table 1. Cyclin D1-CDK4 inhibition activity of cinnamaldehydes

Compound	Inhibition[a]	Compound	Inhibition[a]
Ia (2′-OH)	< 10	**IIb** (3′-OH)	40
IIa (2′-OH)	50	**IIc** (2′-Cl)	51
IId (2′-NO_2)	30		
IIIa (2′-OCH_2Ph)	85	**IIIa′** (3′-OCH_2Ph)	94
IIIb (2′-OCH_2Ph-2-CH_3)	70	**IIIb′** (3′-OCH_2Ph-2-CH_3)	73
IIIc (2′-OCH_2Ph-2-CN)	22	**IIIc′** (3′-OCH_2Ph-2-CN)	45
IIId (2′-$OCCH_2$Ph-2-OCH_3)	43	**IIId′** (3′-OCH_2Ph-2-OCH_3)	65
IIIe (2′-OCH_2Ph-4-CH_3)	68	**IIIe′** (3′-OCH_2Ph-4-CH_3)	84
IIIf (2′-OCH_2Ph-4-Br)	71	**IIIf′** (3′-OCH_2Ph-4-Br)	88
IIIg (2′-OCH_2Ph-4-OCH_3)	59	**IIIg′** (3′-OCH_2Ph-4-OCH_3)	82
IIIh (2′-OCH_2Naphthalene)	38	**IIIh′** (3′-OCH_2Naphthalene)	85
IIIi (2′-$OCH(CH_3)$Ph)	63	**IIIi′** (3′-$OCH(CH_3)$Ph)	77
IIIj (2′-OCH_2CH_2Ph)	72	**IIIj′** (3′-OCH_2CH_2Ph)	80
IIIk (2′-OC(O)Ph-4-F)	25	**IV** (3′-OH)	40
IIIl (2′-OC(O)Ph)	52	**IVa** (3′-OCH_2Ph-4-Br)	58
IIIm (2′-OC(O)Ph-4-CH_3)	50	**V**	54
IIIn (2′-OC(O)Ph-2-OCH_3)	23	**VI**	53
		VII	34

[a]% Inhibition at 5 μg/mL.

Table 2. IC_{50} values for cyclin D1-CDK4 and cyclin B-CDK1

Compound	IC_{50} (μM) (cyclin D1-CDK4)	IC_{50} (μM) (cyclin B-CDK1)
IIa	35	130
IIb	45	30
IIIa	11	50
IIIa′	7.5	50
IIIb	18	200
IIIb′	15	60
Flavopiridol	0.1[a]	

[a]Positive control.

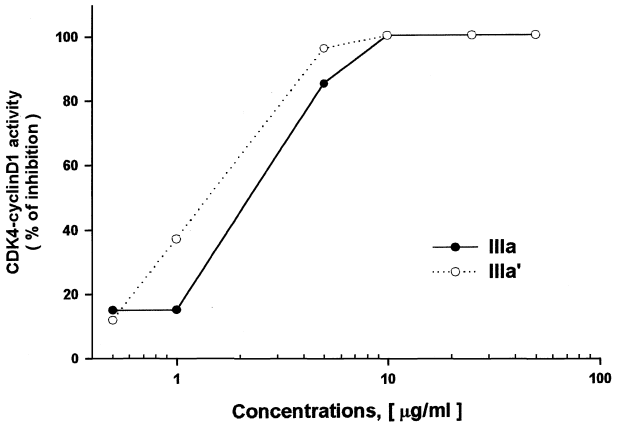


Figure 1. CDK4 inhibition activities by compound **IIIa** and **IIIa′**.

cinnamaldehydes exhibit higher inhibition activity than that of the 2′-substituted compounds towards cyclin D1-CDK4 (see Figure 1). When a polar substituent is introduced at the C2 position of the benzyl group, the inhibitory activity is also significantly decreased. Some compounds with bulky groups such as methoxy-coumarin (**VI**) and anthraquinone (**VII**), and with disubstituted cinnamaldehyde (**IV**) and pyridyl groups (**V**) were also prepared for comparison of the inhibitory activity with the other cinnamaldehydes. We found that a significant loss of inhibitory activity is observed from those compounds. Compounds **IIIb′**, **IIIf′** and **IIIh′** have a high potency for cyclin D1-CDK4 inhibition with IC_{50} values of 7.5, 8.2, and 7.8 μM, respectively. However, these compounds have significantly weaker inhibition activity for the cyclin E-CDK2 (IC_{50} >200 μM) (data not shown).

In a cyclin B-CDK1 inhibition assay, the synthesized compounds exhibited low inhibitory activity compared to cyclin D1-CDK4. Compounds **IIIb** and **b′** show 10 and 8 times the selectivity. The IC_{50} values of the compounds are summarized in Table 2.

In conclusion, a series of cinnamaldehydes has been synthesized to identify the antitumor mechanism of the compound and characterized as CDK4 inhibitors. The most potent of this series, compound **IIIa′**, has been identified. These results should provide valuable information for the design of CDK4 inhibitor and evaluation of biological activities of cinnamaldehydes.

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11. Data for compound **IIIb**: HRMS: 252.1139 (calcd 252.1150 for $C_{17}H_{16}O_2$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.65 (d, 7.5 Hz, 1H), 7.89 (d, 16.3 Hz, 1H), 7.58 (m, 1H), 7.42 (m, 2H), 7.25 (m, 2H), 7.05 (m, 2H), 6.76 (dd, 7.5, 16.2 Hz), 5.09 (s, 3H), 2.38 (s, 3H). Data for compound **V**: HRMS: 132.0441 (calcd 132.0449 for C_8H_6NO). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.74 (d, 7.6 Hz, 1H), 8.90 (s, 1H), 8.64 (d, 4.8 Hz, 1H), 8.17 (d, 8.0 Hz, 1H), 7.75 (d, 16.1 Hz, 1H), 7.48 (dd, 4.8, 8.0 Hz, 1H), 6.90 (dd, 7.5, 16.1 Hz).
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14. Enzyme purification. GST-cyclin D1-CDK4 or GST-cyclin E-CDK2 constructs in baculovirus were expressed in sf21 cells. Cell lysates were subjected to glutathione chromatography to obtain enzymatic purity and activity suitable for kinase assay screening of the compound library. Kinase assay. Enzyme assays to screen for inhibitors of cyclin D1-CDK4 were performed in 96 well plates. Each reaction contained

5 μM GST-RB fusion protein corresponding to the amino acid postion 792–928 of the RB protein, 100 μM ATP, 0.5 μCi [γ-^{33}P] ATP, and 200 nM enzyme in a final volume of 100 μL/well. Compounds were added to the reaction mixture and after 30 minutes at 30°C, the reaction was stopped by addition of ice cold 30% TCA and the phosphorylated products were adsorbed onto PVDF membranes in 96 well microtiter filter plates (Millipore). The radioactivity in [γ-^{33}P] -labeled RB was measured in a Wallac Microbeta Counter after addition of 25 μL/well scintillant (Wallac Optiphase). cyclin E-CDK2 assay was performed with histone H1 for the substrate in the same way as for cyclin D1-CDK4. Cyclin B-CDK1 was purified from starfish oocytes and assayed for 10 min, at 30 °C, at a final ATP concentration of 15 μM as described previously (ref 12).

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