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Novel synthetic chalcones induce apoptosis in the A549 non-small cell lung cancer cells harboring a KRAS mutation

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Abstract

A series of novel chalcones were synthesized by the Claisen-Schmidt condensation reaction of tetralones and 5-/6-indolecarboxaldehydes. Treatment of human lung cancer cell line harboring Kras mutation (A549) with the chalcones induced dose-dependent apoptosis. Cell cycle analyses and Western blotting suggested the critical role of the chalcones in interrupting G2/M transition of cell cycle. SAR study demonstrated that substituent on the indole N atom significantly affects the anticancer activity of the chalcones, with methyl and ethyl providing the more active compounds (EC₅₀: 110–200 nM), Compound **1g** was found to be >4-fold more active in the A549 cells (EC₅₀: 110 nM) than in prostate (PC3) or pancreatic cancer (CLR2119, PAN02) cells. Furthermore, compound **1l** selectively induced apoptosis of lung cancer cells A549 (EC₅₀: 0.55 μM) but did not show measurable toxicity in the normal lung bronchial epithelial cells (hBEC) at doses as high as 10 μM, indicating specificity towards cancer cells.

Graphical Abstract

Keywords

Chalcone; Cell cycle; Apoptosis; KRAS; Non-small cell lung cancer

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Intrinsic and acquired resistance to existing therapies is the major cause of treatment failure for late-stage cancer patients, which necessitates the discovery of novel agents to overcome drug resistance in cancer. Despite decades of intensive academic and industrial research efforts, we still lack drugs that are effective in treating cancers that harbor the KRAS mutation. KRAS mutations are identified in 97%, 44%, and 30% in pancreatic cancer, colorectal cancer, and non-small cell lung cancer (NSCLC), respectively.¹ KRAS mutants are resistant to molecular therapies that target the epithelial growth factor receptor (EGFR). Lung cancer patients bearing the KRAS mutation are mostly treated with standard chemotherapy and radiation. Treatment-induced resistance occurs frequently and renders only short-term benefits. As such, new strategies are needed for KRAS lung cancer patients, who become refractory to existing therapeutics.

A number of approaches have led to small molecule inhibitors that directly target the KRAS GTP/GDP binding domain or its effectors. However, due to the complex signaling pathways that KRAS controls as well as intracellular location of the Ras proteins, the development of KRAS targeted therapeutics still remains a work-in-progress.¹⁻²

Chalcone is a key structural moiety occurring in a number of natural products known to have broad biological activities, including curcumin³ and xanthohumol.⁴⁻⁵ It has gained considerable interests in the synthesis of novel anticancer agents with diverse mechanisms of action.⁶⁻¹⁴

We had previously described indole and indazoline series of anticancer agents capable of overcoming multi-drug resistance¹⁵⁻¹⁸. Herein we report the identification and preliminary structure-activity relationship (SAR) findings of novel indolyl-tetralone chalcones in the A549 NSCLC cell line, a widely used *in vitro* model system for investigating KRAS mutation. Our survey of published literature indicated that there were no known chalcones that contain the same key moieties of tetralone and 5-/6-indolyl group.¹⁹⁻²⁸ We report that a number of the novel chalcones were highly potent in inducing apoptosis of A549 cells, with sub- μ M EC₅₀, which are not commonly observed in reported synthetic chalcones. Among them, compound **11** demonstrated preferential cytotoxicity in A549 cells (EC₅₀: 0.55 μ M) and did not show any toxicity in the normal lung epithelial cells at concentrations ranging from 0.01 to 10 μ M.

The indolyl-tetralone chalcones (**1** and **2**) were synthesized by following the well-established Claisen-Schmidt condensation reaction between a tetralone and an Indole-5-/6-carboxaldehyde (Scheme 1).²⁹ For preliminary structure-activity relationship (SAR) analyses, from commercially available tetralones and 5/6-indolecarboxaldehydes we synthesized 21 chalcones that are structurally distinct from known chalcones (Table 1). All the new compounds were characterized by ¹H NMR and MS spectra and their purity is >90% by reverse phase HPLC analyses.³⁶ In most cases, the products precipitated from the react solution and were isolated by filtration and washing with cold water and ethanol. Typically the isolated yields ranged from 60% to 90%. The yields might reflect the solubility of the product in the reaction media, and no further efforts were deployed to recover the soluble products from the reaction solution. The derivatives with an indole N-BOC (**1d** and

1i) or N-Acetyl (**1h**) were prepared by treating their corresponding indole NH analogs (**1a** or **1f**) with Boc₂O or Ac₂O, respectively.

We first tested the compounds at 1 and 10 μ M in the A549 cell lines and then determined the EC₅₀ of those compounds showed significant activity at both concentrations. A549 cells were treated with the inhibitors at various concentrations for 72h. Cells were stained with crystal violet and absorbance corresponding to number of cells alive was evaluated at 562 nm. As shown in Table 1, the indole isomers (e.g. **1b** vs. **2a**) have minimal affect on the activity. However, the indole nitrogen (N) position is more sensitive to substitutions (R³). Derivatives with NH, NCH₃, and NC₂H₅ are very active (e.g. **1a–c**), while those with the bulky benzyl (**1j**) and BOC (**1d**, **1i**) groups are inactive even at 10 μ M. The electron-withdrawing acetyl (COCH₃) seems to significantly decrease the activity as well. Therefore, majority of the analogs were synthesized with the indole NH or substituted with CH₃ or C₂H₅. Overall, the NCH₃ and NC₂H₅ analogs are among the most active compounds.

Interestingly, the substituents on the tetralone moiety profoundly affect the anticancer activity of the chalcones. Thus, compounds substituted with fluorine (F-) at the 7-position of the tetralone are more potent than their corresponding unsubstituted analogs (e.g. **1e–g** vs. **1a–c**). The effect of the F- substitution is most significant between **1a** and **1f** with a >4-fold difference in their activity. On the other hand, a bromine (Br-) at the same position (e.g. **1p–q**) seems to reduce the activity significantly, leading to only weakly active compounds. Furthermore, **1l** is the best among the compounds substituted with a mono-methoxy (MeO-) group, with an EC₅₀ of 0.55 μ M. Derivatives with di-methoxy groups (**1n**, **1o**, **2c**) are at best only marginally active at 10 μ M dose.

As we described recently³⁰, the chalcone analog **1g** was effective in inducing apoptosis of PC3 prostate cancer, as well as the CRL-2119 (human) and PAN02 (murine) pancreatic cancer cell lines. Compound **1g** dose-dependently decreased the number of viable cells after 72 hours treatment. The EC₅₀ of **1g** was determined as 0.45, 0.31, and 0.59 μ M in PC3, CRL-2119, and PAN02 cell lines, respectively, indicating a preferential cytotoxicity for **1g** in A549 cell line (EC₅₀: 0.11 μ M). On the other hand, the chalcone **1l** seemed to be preferentially cytotoxic against the A549 lung cancer cells. Thus, **1l** potently and dose-dependently induced apoptosis in A549 cancer cell line with an EC₅₀ of 0.55 μ M. On the other hand, its anticancer activity in the prostate and pancreatic cancer cell lines was considerable less potent than in the A549 cells. At 1 μ M concentration, it induced less than ~40% cancer cell deaths in PC3, CRL-2119, and PAN02 cancer cell lines as compared to ~80% of A549 cell death. More importantly, **1l** was found to be much less toxic in the normal human bronchial epithelial cells (hBEC) (Figure 1). At the dose range of 0.1–10 μ M, **1l** did not induce measurable changes of cell viability in hBEC cells. Therefore, the *in vitro* therapeutic index of **1l** is >18-fold between A549 and hBEC cells.

To evaluate the mechanism of the chalcones' anti-cancer activity containing indolyl group, we performed cell cycle and cell death analyses in the same cancer and normal cell lines as above. For comparison, we also prepared a chalcone (**3**) where the indole was replaced by a trimethoxyphenyl group, a key structural feature that occurred in a number of synthetic and natural products with anticancer activities, including combretastatin A4 and

colchicine.^{31–35} Similar compounds were known to induce cell cycle arrest and apoptosis of cancer cells.³⁶

(3)

Cells were starved for 24h without serum to induce G1/G0 blockade. Cells were then treated with medium with FBS and chalcone derivatives (1 μ M) were added to the medium for another 24h. Treated cells (0.5×10^6) were processed with propidium iodide staining and then analyzed by FACS Calibur flow cytometer and Cellquest software. The treatment of A549 cells with inodyl chalcone **11** induced G2/M transition blockage and was associated with increase in apoptosis likely due to induction of mitotic catastrophe (Figure 2A). **11** did not alter cell cycle of human bronchial epithelial cells (hBEC), further demonstrating the low toxicity of the compound on normal epithelial cells (Figure 2B). The differential effect on normal versus cancer cells might be explained by the higher proliferation rate and abnormal cell cycle (i.e. failure of checkpoints) in cancer cells. The proliferation of normal hBEC cells is under tight cell cycle regulation, which renders them less sensitive to the indole chalcones that modulate key regulators of cell cycle progression (see below). In contrast, chalcone **3** without the inodyl group induced cell cycle blockage in G1 phase of cell cycle and inhibition of S phase in PC3 cells but not in A549 cells (Figure 2C). This data indicates the unique role that the indole moiety plays in inducing G2/M phase cell cycle blockade.

Further, we performed Western blotting assessing the expression of cell cycle proteins to explain the molecular mechanism behind our observation. Upon addition of serum containing medium, A549 cells showed increases in cyclin A2, Retinoblastoma protein (Rb) phosphorylation (a marker of proliferation) and phosphorylated stathmin (P-STMN1), which were indications of cell cycle entry and progression. We showed that cyclin A2 and phosphorylated Rb, but not cyclin B1, were suppressed by **11** (Figure 3). Strong blockage of P-Rb might be directly linked to low cyclin A2 expression upon treatment with **11**. Low levels of cyclin A2 prevent cancer cells from entering mitosis and lead to G2/M phase blockage after treatment with **11** (Figure 3 and 2A). Similar to our previous data with chalcones derivatives³⁰, we showed that STMN1 is phosphorylated early upon treatment with **11** (8h). This data indicates that STMN1-regulated microtubule assembly and progression of cell cycles are the likely targets of **11** in A549 lung carcinoma.

In summary, we described the SAR studies of a series of novel synthetic chalcones. Our data support the exploration of the indolyl-tetralone chalcones as a new structural template for the discovery of anticancer drugs for cancer harboring KRAS mutation as A549 lung cancer cells. Cell cycle analyses suggested the critical role of the indole chalcones in interrupting late phases (G2/M) of cell cycle rather than cell cycle blockage in G1. It is noteworthy that certain analogs, such as **11**, are much more active in KRAS mutant A549 lung cancer cells than normal lung epithelial cells. We had reported previously our findings with regarding to their novel mechanism of targeting selectively the stathmin protein that plays critical role in microtubule dynamics and mitotic catastrophe. Taken together, our results reveal an attractive structural template for the discovery of mechanism based novel anticancer agents.

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37. **Synthesis of (2E)-7-fluoro-2-[(1-methyl-1H-indol-5-yl)methylidene]-1,2,3,4-tetrahydronaphthalen-1-one (1e).** Sodium hydroxide (8M, 0.6 mL) was added to a mixture of 7-fluoro-1-tetralone (82 mg, 0.5 mmol) and N-methyl-indole-5-carbaldehyde (80 mg, 0.5 mmol) in three (3) mL of EtOH and stirred at room temperature overnight. The resulting precipitates were collected via filtration, and washed with EtOH and water to yield compound 1e as a fine yellow solid in 90% yield. ¹H NMR (400MHz, DMSO-d₆): δ 2.91–2.95 (m, 2H), 3.17–3.20 (m, 2H), 3.28 (s, 3H), 6.52 (d, *J* = 3.2 Hz, 1H), 7.36–7.44 (m, 4H), 7.52 (d, *J* = 8.8 Hz, 1H), 7.63 (dd, *J* = 8.8, 1.6 Hz, 1H), 7.80 (s, 1H), 7.90 (s, 1H). ¹³C NMR (100 MHz, acetone-d₆): δ 185.7 (d, *J*_{C-F} = 8.8 Hz), 162.9, 160.5, 139.3 (d, *J*_{C-F} = 12.4 Hz), 138.8, 137.0, 135.4 (d, *J*_{C-F} = 24.4 Hz), 132.0, 130.5 (t, *J*_{C-F} = 30.4 Hz), 128.7, 126.6, 123.9, 123.1, 119.8 (d, *J*_{C-F} = 88 Hz), 113.0 (d, *J*_{C-F} = 88 Hz), 109.5, 101.4, 32.1, 28.5, 28.3. ESI(+) MS *m/z* calc'd for C₂₀H₁₆FNO: 305.35 found: 306.3 (M + H)⁺. HRMS (ESI) *m/z* calc'd for C₂₀H₁₆FNO: 305.1294. found: 306. 1302 (M + H)⁺.

Figure 1. Dose-dependent effect of chalcone 11 on cell survival of cancer cells and normal epithelial cells

Cells were treated with **11** or DMSO (controls) for 72 hours at concentrations of 0.033–10 μ M, followed by crystal violet staining. E. Crystal violet staining of normal bronchial epithelial cells (hBEC), prostate cancer cells (PC3), lung carcinoma (A549), pancreatic (#CLR2119- HPAC, PANC02) cell lines treated with **11** or DMSO at various concentrations for 72h. Cells were stained with crystal violet and absorbance corresponding to number of alive cells was evaluated at 562 nm. Data are representative for n=6 performed twice. Averages $\pm$ SD are shown.

Figure 2. Synthetic chalcones induce cell death via differential effects on cell cycle
 Cell cycle analysis of propidium iodide (PI) stained A549 (A) or hBEC (B). Number of cells gated in G1, S or G2/M as well as apoptotic and necrotic cells is shown (C). *: p value <0.05. Data are representative for n=4–5 repeats.

Figure 3. Immunoblotting with antibodies against cell cycle regulators cyclin A2, Cyclin 1, P-Rb protein, total Rb and P-STMN1 in A549 cells treated with vehicle (DMSO) or chalcone **11** (1 μ M) for 8, 24, 48h. Data are representative for 2 independent experiments.

Scheme 1.

Claisen-Schmidt condensation reaction for the synthesis of indolyl-tetralone chalcone **1e**.
condition: a. NaOCH₃ in methanol; 90%.

Table 1

Indolyl-tetralone chalcone derivatives produced via Scheme 1 and their cytotoxicity in A549 cancer cell line

Entry	Code	R ¹	R ²	R ³	EC ₅₀ (μM) (% of apoptotic cells at 10 μM) <i>a</i>
		1
		2			
	1a	H	H	H	1.13	
	1b	H	H	CH ₃	0.47	
	1c	H	H	C ₂ H ₅	0.46	
	2a	H	H	CH ₃	0.47	
	1d	H	H	BOC	(N.A.) <i>b</i>	
	1e	F	H	CH ₃	0.20	
	1f	F	H	H	0.27	
	1g	F	H	C ₂ H ₅	0.11	
	1h	F	H	CH ₃ CO	(28)	
	1i	F	H	BOC	(N.A.)	
	1j	F	H	Bn	(N.A.)	
	1k	CH ₃ O	H	C ₂ H ₅	3.0	
	1l	CH ₃ O	H	H	0.55	
	1m	CH ₃ O	H	CH ₃	3.0	
	2b	CH ₃ O	H	CH ₃	5.8	
	1n	CH ₃ O	CH ₃ O	CH ₃	(N.A.)	
	1o	CH ₃ O	CH ₃ O	C ₂ H ₅	(19)	
	2c	CH ₃ O	CH ₃ O	CH ₃	10	
	1p	Br	H	H	(16)	
	1q	Br	H	CH ₃	(21)	
	2d	Br	H	CH ₃	(26)	
	1r	Br	H	C ₂ H ₅	(N.A.)	

average of triplicates, % of apoptotic cells in treatment groups as compared to DMSO controls.

^a N.A.: not active at 10 μ M; Bn: benzyl; BOC: t-butoxycarbonyl. Docetaxel was used as a positive control and consistent with our historic results.