Published in final edited form as:

Bioorg Med Chem Lett. 2011 January 1; 21(1): 555–557. doi:10.1016/j.bmcl.2010.10.063.

Chalcone-based inhibitors against hypoxia-inducible factor 1 – Structure activity relationship studies

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Abstract

Many tumor tissues are under hypoxic conditions. Activating hypoxia-inducible factor 1 (HIF-1), a transcription factor, is a major mechanism for tumor cells to survive and even to evade other tissues. Therefore inhibiting HIF-1 is a potential strategy to help improve cancer treatment. Chalcone is a promising template to develop HIF-1 inhibitor because quite a few of chalcone-based compounds reveal moderate HIF-1 inhibitory activity and many chalcone-based compounds demonstrate promising anticancer activities in various animal models. However, there are no reports about the structure-activity relationship of chalcone compounds with respect to HIF-1 inhibition. This study reports the HIF-1 inhibitory activities of a panel of chalcones, identifies a few lead candidates of single-digit micromolar potency, and determines important structural modifications.

Hypoxia-induced factor 1 (HIF-1) is a heterodimeric transcription factor that is composed of a constitutively expressed HIF-1 β subunit and an O₂-regulated HIF-1 α subunit. HIF-1 α subunit is typically polyubiquitinated and proteasomal degraded under non-hypoxic conditions, leading to HIF-1 suppression. Under hypoxic condition, HIF-1 α degradation is inhibited and intact HIF-1 α dimerizes with HIF-1 β , activating HIF-1 regulated transcription.

¹ Due to the high proliferation of tumor cells and the lack of adequate blood vessel development, many tumors are under hypoxic conditions. Functionally, HIF-1 regulates hundreds of genes at the transcription level, many of which play key roles in critical processes of cancer development, including angiogenesis, cell survival, invasion and metastasis.² HIF-1, therefore, is a potential drug target for cancer treatment.³

Although quite a few of standard cancer chemotherapies have been reported to inhibit HIF-1 activity, it remains to be determined whether their HIF-1 inhibitory activities are indirect or off-target effects.⁴ Currently there are only a limited number of small molecules originally designed or discovered as HIF-1 inhibitors,⁵ including a few chalcone-based compounds – kuwanons (Fig. 1).⁶ No structure-activity relationship has been carried out on any of these lead compounds. The chalcone-based kuwanon compounds are highly complex in structure that it remains to be determined if simplified chalcone may still recapitulate the HIF-1 inhibitory activity. Chalcone is a privileged template to develop HIF-1 inhibitor because quite a few chalcone-based candidates, natural or synthetic, have demonstrated promising anticancer activities in various animal models (Fig. 2).^{7–13} For instance, a simple chalcone-based natural product, isoliquiritigenin, suppresses pulmonary metastasis of mouse renal cell

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carcinoma⁸ and effectively prevents colorectal tumor development at a dose of 100 ppm.^{9,10} Nevertheless the actual mechanism of action of chalcone-based compounds for their anticancer activities have not been well established.¹⁴

We have recently synthesized several series of simple chalcone-based compounds; all these candidates can be synthesized in no more than three steps with >50% overall yield.¹⁴ Given the shortage of HIF-1 inhibitors, particularly with simple chemical structure and easy synthetic access, and chalcone being a potential template to develop such inhibitors, we evaluated the simple chalcone candidates we have synthesized before for their HIF-1 inhibitory activities. This study is also a continuous effort for us in elucidating the molecular basis for the anticancer activity of chalcone-based compounds.

Using an *in vitro* luciferase-based assay with a NIH3T3 cell line stably transfected with HIF-1-luciferase, we have evaluated two series of chalcone compounds for their HIF-1 inhibitory activity.¹⁵ Curcumin was used as a positive control for comparison as it was shown to be a potent HIF-1 inhibitor and it shares certain structural similarity to chalcone compounds, particularly the enone functionality.¹⁶ The first series have substituents of varied electron density on both rings with a goal to explore whether the electron density on chalcone may influence the enone's electrophilicity (Table 1, 1a–1i), which may be potentially responsible for the HIF-1 inhibitory activity. The second series have the same substituents on the A ring as three chalcone-based natural products – flavokawain A, B, and C.¹⁷ These analogs have varied substituents on the B ring (Table 2, 2a–2k) with a goal to determine whether modification of B ring could affect their HIF-1 inhibitory activities.

Chalcone-based compounds were first evaluated at a single concentration of 20 μ M for their inhibitory activity against cobalt chloride-induced HIF-1 activation. Candidates with > 50% inhibitory activities at 20 μ M were further evaluated in a dose-dependent manner to determine the concentration needed to inhibit 50% of cobalt chloride-induced HIF-1 activation (IC_{50}). As shown in Table 1, most of the chalcones in Series 1 showed no HIF-1 inhibitory activities irrespective of the electron density in the chalcone system (IC_{50} > 20 μ M), except **1c** (isoliquiritigenin). **1c** demonstrated a decent HIF-1 inhibitory activity with an IC_{50} of 4.8 μ M, twice as potent as curcumin – the positive control. *o*-hydroxy substitution at alkene side of phenyl ring of the chalcone structure as in **1b** do not confer activity (IC_{50} > 20 μ M). Butein (**1d**) which has an extra hydroxy substitution at *m*-position at alkene side of phenyl ring of the chalcone structure when compared to **1c** is also inactive (IC_{50} > 20 μ M). This clearly reveals that hydroxy substitution at *p*-position at alkene side of phenyl ring of the chalcone structure is important for activity and might be involved in hydrogen bonding interaction with the protein target. As the hydroxy substitutions at *m*- and *p*-positions at alkene side of phenyl ring of the chalcone structure of **1d** may form intramolecular hydrogen bonding, the crucial *p*-hydroxy group needed for activity may not be available for hydrogen bonding interaction with protein target; and this can explain complete lose of activity of **1d**. The inhibitory potency of **1c** is also comparable to that from kuwanons, the much more complex chalcones.⁶ Interestingly, kuwanon R (IC_{50} = 3.2 μ M), which appears to be the most potent HIF-1 inhibitors among the four kuwanon candidates, happens to have the same regio hydroxy substitutions on the chalcone system as **1c** does, suggesting that the position of the hydroxy functional groups may be critical as well. More interesting, among the chalcone candidates in Table 1, **1c** is the only one that has been widely demonstrated as a promising anticancer agent,^{8–10} suggesting that HIF-1 inhibition may be involved for its anticancer activity.

Nevertheless, multi hydroxy functional groups on **1c** may significantly increases its rate of metabolism and/or prevents its oral bioavailability to certain tissues,¹⁸ chalcones with less hydroxy functional groups while retaining the HIF-1 inhibitory activity could be

complementarily important. We therefore evaluated chalcones with the same A-ring as flavokawains as shown in Series 2 (Table 2) for their HIF-1 inhibitory activity, many of which have only one hydroxy functional group. Excitingly, four candidates demonstrate decent HIF-1 inhibitory activities ($2\ \mu\text{M} < \text{IC}_{50} < 5\ \mu\text{M}$) with **2g** being the most potent with an IC_{50} of $2.2\ \mu\text{M}$. The R^1 position seems to accommodate small non-halogen substituents, such as H or OH (**2b** and **2c**) while large substituents led to 4-fold decrease in potency (**2a** and **2d**) and halogen substituents led to complete loss of activity (**2e** and **2f**). The R^2 position seems to tolerate substitution (**2g** and **2h**) although such modifications do not change the activity significantly. The R^3 position on the other hand does not tolerate substitution as **2i** and **2j** are 3–4 fold less potent compared to **2b**. Lastly, R^4 position does not accommodate modification that a CH_3 modification (**2k**) led to complete loss of activity relative to **2b**.

In summary, four HIF-1 inhibitors were identified from simple chalcones that can be readily synthesized in one to two steps. These inhibitors reveal comparable HIF-1 inhibitory activity relative to the complex kuwanons, confirming that structural complexity on the chalcone template as seen in kuwanons is not required to obtain HIF-1 inhibitory activity. Our preliminary structure-activity relationship studies also reveal positions on the chalcone framework that can be modified or substituted. Works are ongoing to evaluate the anticancer activity of the lead compounds and to elucidate the mechanisms of chalcone-based HIF-1 inhibition.

Acknowledgments

This investigation was partially supported by Grant R03CA125844 from National Cancer Institute, NIH (C. Xing).

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15. HIF-1 inhibitory assay: NIH3T3 cell line stably transfected with HIF-1-luciferase was purchased from Panomics (Cat # RC0017, Fremont, CA). This cell line was obtained by co-transfecting NIH3T3 cells with pHIF-1-luc and pHyg followed by hygromycin selection, which can monitor HIF-1 activity *in vitro*. This cell line was cultured using DMEM medium supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 $\mu\text{g/ml}$), and hygromycin (100 $\mu\text{g/ml}$) at 37°C

with 5% CO₂. Cells were plated in a 96-well plate at a density of 5,000 cells/well. Twenty-four hours after plating, the cell medium was replaced with FBS-free fresh medium and incubated at 37°C with 5% CO₂ for 1 hour to minimize the potential stimulation to the cells induced by medium change. Cells were then treated with cobalt chloride (150 μM) and chalcone-based compounds simultaneously for 8 hours. Cells treated with cobalt chloride alone served as positive controls while cells without cobalt chloride treatment served as negative controls. Luciferase activities from these cells were then measured by using the Bright-Glo Luciferase Assay Kit from Promega (Cat # E2620, Madison, WI), following manufacture's protocol (Cat #TM052). The relative HIF-1 activities of the cells treated by chalcone candidates were obtained as the ratio of its luciferase activity to that from the positive controls, both of which have been corrected with background (signals from negative controls) and cell viability.¹⁹ Under these experimental conditions, none of the chalcone compounds induced significant toxicity to NIH3T3 cells (< 5% reduction of cell viability). The IC₅₀ of each fraction was determined by fitting the relative HIF-1 activity to the drug concentration by using a sigmoidal dose-response model of varied slope in GraphPad. The IC₅₀ reported herein is the average of at least three replicates with standard error.

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Fig. 1.
Kuwanon J, Q, R, and V as HIF-1 inhibitors.

Fig. 2.
Representative chalcones with *in vivo* anticancer activities.

Table 1

HIF-1 inhibitory activities^a of chalcones with substituents of varied electron densities on A and B rings.

	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶	IC ₅₀ ± SD (μM)
1a	H	H	H	H	H	H	> 20
1b	H	H	OH	H	H	H	> 20
1c	OH	H	H	OH	H	OH	4.8 ± 1.2
1d	OH	OH	H	OH	H	OH	> 20
1e	H	H	H	CH ₃	H	H	> 20
1f	H	H	H	F	H	H	> 20
1g	H	H	H	CF ₃	H	H	> 20
1h	H	H	H	F	F	H	> 20
1i	OH	H	H	H	H	H	> 20
curcumin							9.6 ± 1.2

^a Results are given as the mean of three independent experiments with triplicate in each experiment.

Table 2

HIF-1 inhibitory activities^a of chalcones with the same A-ring as flavokawains

	R ¹	R ²	R ³	R ⁴	IC ₅₀ ± SD (μM)
2a	OCH ₃	H	H	H	11.0 ± 2.6
2b	H	H	H	H	3.3 ± 1.1
2c	OH	H	H	H	3.1 ± 1.2
2d	OCH ₂ CH ₂ CH ₃	H	H	H	12.0 ± 4.9
2e	F	H	H	H	> 20
2f	Cl	H	H	H	> 20
2g	H	Br	H	H	2.2 ± 0.4
2h	H	CH ₃	H	H	3.6 ± 0.5
2i	H	H	Br	H	11.6 ± 0.7
2j	H	H	CH ₃	H	9.9 ± 4.8
2k	H	H	H	CH ₃	> 20

^aResults are given as the mean of three independent experiments with triplicate in each experiment.