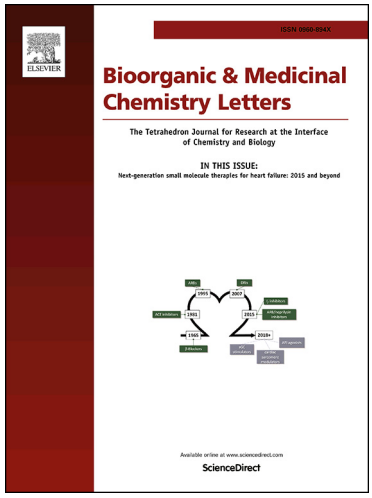
# Accepted Manuscript




Synthesis and biological evaluation of Complex I inhibitor R419 and its derivatives as anticancer agents in HepG2 cells

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## Graphical Abstract

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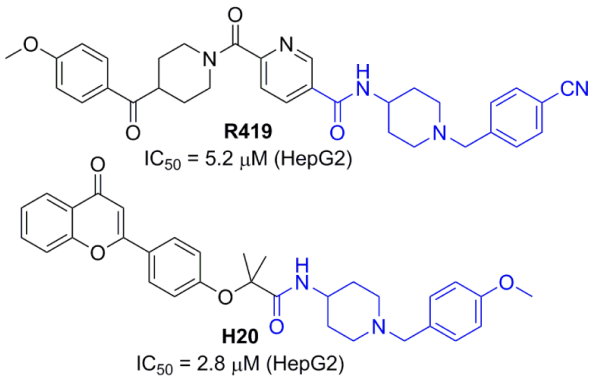


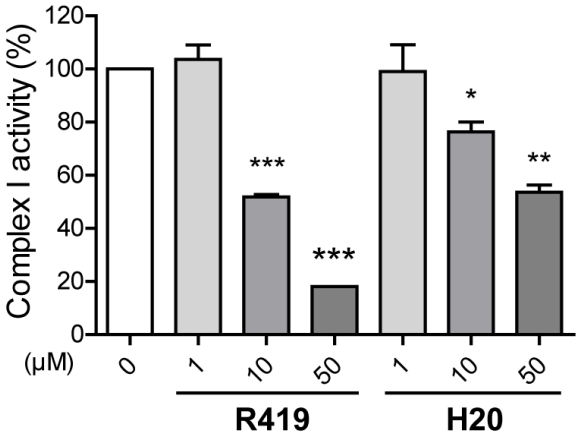

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# Synthesis and biological evaluation of Complex I inhibitor R419 and its derivatives as anticancer agents in HepG2 cells

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ABSTRACT

In this study, Complex I inhibitor **R419** was firstly revealed to have significant anticancer activity against HepG2 cells ($IC_{50}$ = 5.2±0.9 μM). Based on this finding, a series of **R419** derivatives were synthesized and biologically evaluated. As results, 9 derivatives were found to have obvious anticancer activity. Among them, **H20** exhibited the most potent activity ($IC_{50}$ = 2.8±0.4 μM). Mechanism study revealed that **H20** caused severe depletion of cellular ATP, dose-dependently activated AMPK, decreased Bcl-2/Bax ratio and induced necrotic cell death. Most importantly, **H20** displayed definite inhibitory activity against Complex I.



Cellular ATP for energy consumption in mammalian cells is mainly produced by mitochondrial oxidative phosphorylation (OXPHOS). NADH–ubiquinone oxidoreductase (Complex I) is the first OXPHOS enzyme that mediates mitochondrial electron transport[1] and catalyzes the oxidization of NADH produced in the tricarboxylic acid (TCA) cycle and in the β-oxidation of fatty acids to regenerate $NAD^+$ for the mitochondrial matrix.[2] Although as revealed by Otto Warburg cancers tend to reprogram metabolism to produce energy via glycolysis rather than OXPHOS, some types of cancers still rely on OXPHOS.[3-4] Thus, it is possible to interfere with OXPHOS for treatment of specific cancers. Complex I inhibitors, *e.g.* phenformin, metformin, rotenone and AG311, have been demonstrated to possess anticancer activity.[5-8]

Notably, compound **R419** (Figure 1) was a newly identified Complex I inhibitor that improved glucose and lipid homeostasis in both AMPK-dependent and AMPK-independent fashions.[9] To the best of our knowledge, there was previously no report on the anticancer activity of **R419**. Interestingly, we found that **R419** showed relatively potent inhibitory activity against HepG2 hepatocellular cells ($IC_{50}$ = 5.2±0.9 μM) in comparison to L02 normal liver cell line ($IC_{50}$ = 25.9±6.9 μM) and A549 lung cancer cell line ($IC_{50}$ = 19.5±6.8 μM) (supplementary data). Encouraged by this result, a series of piperidine or piperazine derivatives bearing flavonoid fragments (**H1-H20**, Figure 1) were designed and synthesized to search for more potent anticancer agents. As it is known, flavonoids are abundant in vegetables and fruits,[10] and some flavonoids were reported as potent anticancer active ingredients in herbal medicines.[11-12] Morever, some flavonoids were reported to inhibit Complex I.[13-14] Herein we briefly report our study results.

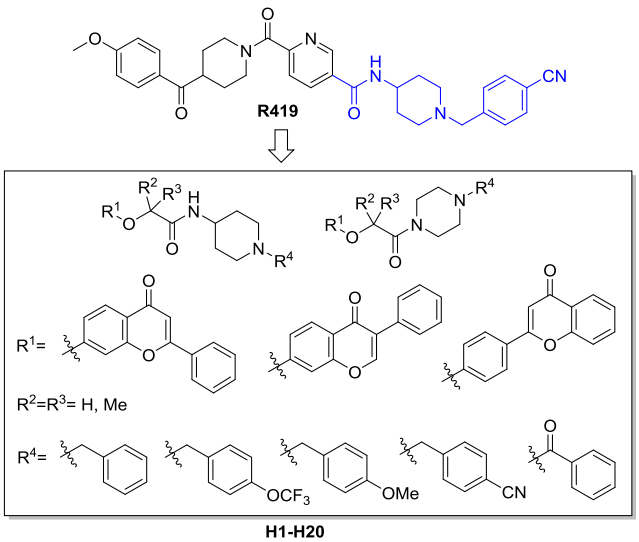
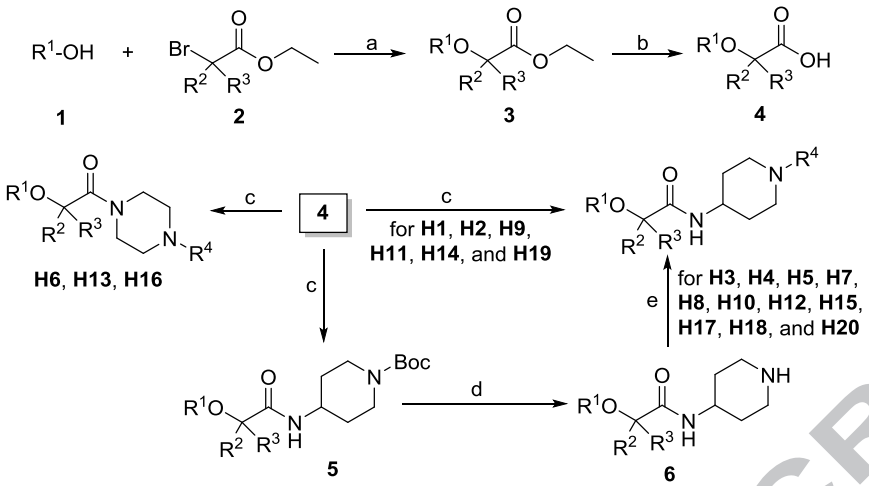



**Figure 1. R419** and designed compounds **H1-H20**.

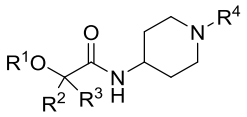



**Scheme 1.** Reagent and conditions: (a) $K_2CO_3$, DMF, 70 °C, 44-86%; (b) NaOH (1N), MeOH/DCM; (c) amines, CDI, DMF, 70 °C, 13-68%; (d) TFA, DCM; (e) $R^4X$, $K_2CO_3$, acetone, 70 °C, 31-88%.

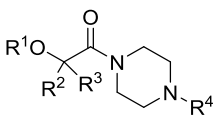
**R419** was synthesized according to the previously reported method.[15] The derivatives **H1**-**H20** were synthesized following the routes shown in **Scheme 1**. Treatment of hydroxyl flavonoids (**1**)[16-18] with 2-bromoacetates (**2**) in the presence of potassium carbonate provided flavonoid esters (**3**), which were hydrolyzed in 1 N sodium hydroxide methanol solution to afford carboxylic acids (**4**). Direct coupling of the carboxylic acids (**4**) with appropriate 1-benzylpiperidin-4-amines or 1-benzylpiperazine amines by CDI in DMF gave piperidine amides (**H1**, **H2**, **H9**, **H11**, **H14**, **H16**, **H19**) or piperazine amides (**H6**, **H13**, **H16**). Alternatively, coupling of **4** with *tert*-butyl 4-aminopiperidine-1-carboxylate was followed by de-Boc reaction in TFA to give piperidines (**6**), which were further alkylated or acylated by diverse benzyl halides or benzoyl chloride to achieve the final targets compounds (**H3**, **H4**, **H5**, **H7**, **H8**, **H10**, **H12**, **H15**, **H17**, **H18**, **H20**).

Compounds **R419** and **H1**-**H20** were evaluated for their anticancer activity against HepG2 cells. The $IC_{50}$ values are summarized in Table 1. **R419** and its 9 derivatives exhibited significant anticancer activity ($IC_{50}$ < 20 μM), among which **H20** displayed the best activity with $IC_{50}$ 2.8±0.4 μM. SAR analysis revealed the following features. Firstly, compounds with 4-substitued benzyl groups were generally more active than those with 4-unsubstitued benzyl (*e.g.* **H3** *vs* **H1**, **H17** *vs* **H14**). Secondly, all the compounds with isoflavone fragment (**H9**-**H13**) were all inactive. Thirdly, all the compounds with a piperazine ring (**H6**, **H13**, **H16**) were also inactive, suggesting that the piperidine ring is probably an important pharmacophore. Moreover, benzoyl as the $R^4$ group resulted in complete loss of activity (**H8**, **H15**). Interestingly, electrical change did not take obvious effect on anticancer activity, as evidenced by that compounds with EWG (electron withdrawing group) and EDG (electron donating group) on the benzyl showed similar activity (*e.g.* **H5** *vs* **H7**, **H17** *vs* **H18**).

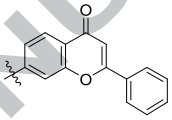
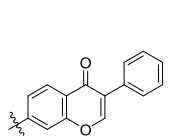
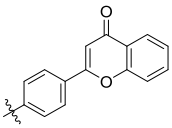
**Table 1.** Cytotoxicity of **R419** and **H1**-**H20** in HepG2 cell line. [a]



**H1-H5**, **H7-H12**, **H14**, **H15**, **H17-H20**



**H6**, **H13**, **H16**

| Cpd | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $IC_{50}$ (μM) [b] |
|---|---|---|---|---|---|
| **H1** |  | H | H | Bn | 16.7±0.1 |
| **H2** | | Me | Me | Bn | 14.0±0.9 |
| **H3** | | H | H | 4-$CF_3$-Bn | 7.2±1.0 |
| **H4** | | Me | Me | 4-$CF_3$-Bn | >20 |
| **H5** | | H | H | 4-OMe-Bn | 8.7±0.8 |
| **H6** | | H | H | Bn | >20 |
| **H7** | | H | H | 4-CN-Bn | 9.3±0.1 |
| **H8** | | H | H | Bz | >20 |
| **H9** |  | H | H | Bn | >20 |
| **H10** | | H | H | 4-OMe-Bn | >20 |
| **H11** | | Me | Me | Bn | >20 |
| **H12** | | H | H | 4-CN-Bn | >20 |
| **H13** | | H | H | Bn | >20 |
| **H14** |  | H | H | Bn | 15.5±0.5 |
| **H15** | | H | H | Bz | >20 |
| **H16** | | H | H | Bn | >20 |
| **H17** | | H | H | 4-CN-Bn | 6.7±1.2 |
| **H18** | | H | H | 4-OMe-Bn | 6.8±0.6 |
| **H19** | | Me | Me | Bn | >20 |
| **H20** | | Me | Me | 4-OMe-Bn | 2.8±0.4 |
| **R419** | | | | | 5.2±0.9 |

[a] HepG2 cells were incubated with vehicle (0.1% DMSO) or compounds for 72 h and cell viability was measured by MTT assay;

[b] The data represented as means ± SD of three separate experiments.

Interestingly, the most potent compound **H20** exhibited good selectivity for HepG2 cell line over L02 normal liver cell line ($IC_{50}$ = 17.6±1.6 μM) and A549 lung cancer cell line ($IC_{50}$ = 40.0±2.7 μM) (Figure 2). We next selected **H20** for further mechanism study. Cell death analysis was performed through flow cytometry by Annexin V-FITC/PI assay to determine whether necrosis or apoptosis dominated the anticancer activity of **H20**. As shown in Figure 3, treatment of HepG2 cells with 0.3 μM and 3 μM of compound **H20** for 40 hours resulted in 11.0% and 46.4% cellular necrosis, respectively, remarkably more than 7.0% in DMSO control group. Through flow cytometry, no apoptosis was observed in cells exposed to **H20**. This was further confirmed by western blot assay of the apoptotic markers, since **H20** did not induce caspase 3 and PARP cleavage in HepG2 cells (supplementary data).

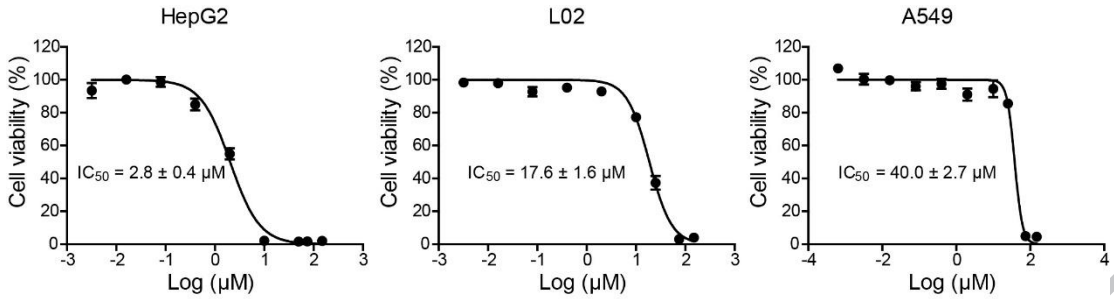



**Figure 2.** Cytotoxicity assay of **H20** in HepG2 cells, L02 cells and A549 cells.

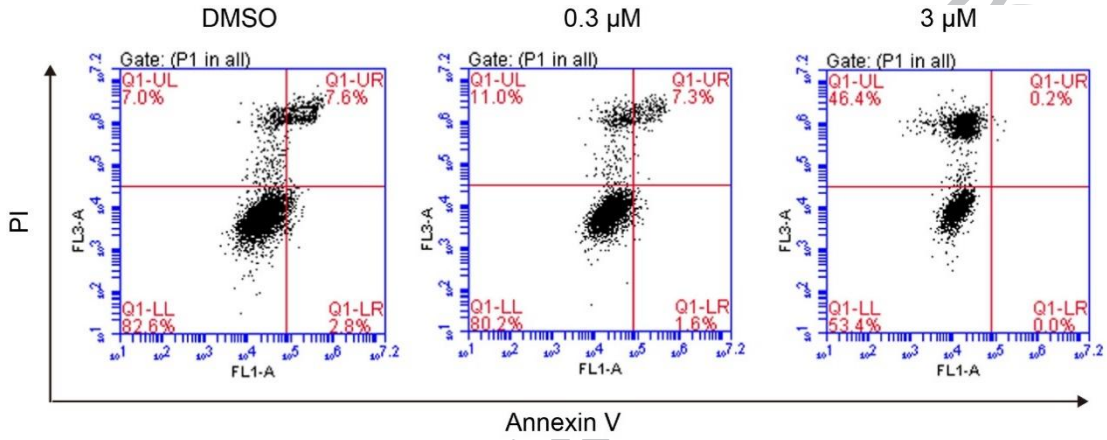



**Figure 3.** Cell death analysis in HepG2 cells exposed to compound **H20** by Annexin V-FITC/PI assay.

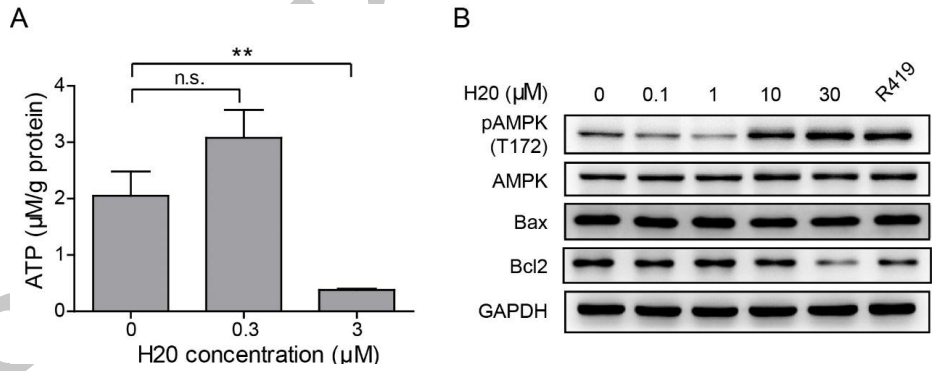



**Figure 4.** (A) Effect of compound **H20** on intracellular ATP levels. (B) Effects of compounds **H20** and **R419** on AMPK activation and Bcl-2/Bax ratio.

In contrast to apoptosis that ATP is required, necrotic cell death is a consequence of ATP depletion.[19] Therefore, effect of **H20** on intracellular ATP levels was measured. It was found that exposure to 3 μM of **H20** for 44 h caused severe depletion of cellular ATP levels, while on the contrary low dose 0.3 μM led to a slight increase despite without statistical significance (Figure 4A). Effects of compound **H20** on AMPK phosphorylation and Bcl-2 family proteins were further studied, as it is known that increase of AMP/ATP ratio can activate AMPK unconditionally[20] and Bcl-2 family proteins play an important role in regulating cell response to lethal signals by governing the mitochondrial membrane permeabilization.[21-22] As expected, **H20** triggered AMPK activation in HepG2 cell in a dose-dependent manner, which at 10 μM seemed as potent as R419 (Figure 4B). Bcl-2 protein level was obviously decreased by **H20**, but Bax not, thus leading to an increase in mitochondrial membrane permeabilization and necrotic cell death at low ATP level. Together, these results indicate that **H20** may cause destruction of mitochondrial energy metabolism.

Mitochondrial oxidative phosphorylation (OXPHOS) not only produces the majority of ATP, but also participates in regulation of cell death.[23] We therefore sought to verify whether **H20**-induced ATP depletion was through Complex I inhibition. **H20**, **R419** and compound **H10** were selected to assess the inhibitory effect on Complex I. As shown in Figure 5, both **H20** and **R419** exhibited dose-dependent and moderate inhibitory activity of Complex I, while **H10** with weak anticancer activity had weak inhibitory activity on Complex I merely at 50 μM. Inconsistent with the *in vitro* cytotoxicity against HepG2 cells, the inhibitory potency of **H20** against Complex I was somewhat lower than that of **R419**, suggesting that **H20**-induced dysfunction of OXPHOS may be through both Complex I-dependent and independent pathways.

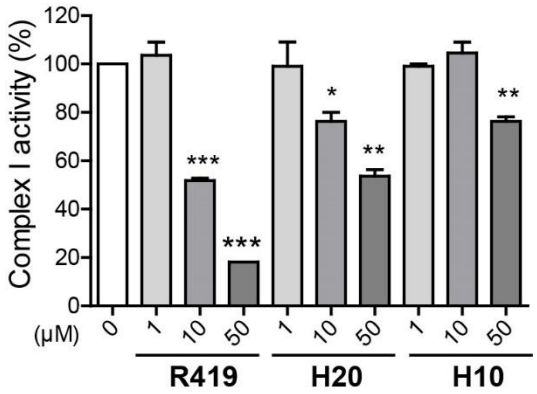



**Figure 5.** Effects of **R419**, **H20** and **H10** on Complex I activity. The Complex I activity values are represented as percentages of the vehicle group. The data are analyzed with Student's *t* test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (compared with vehicle).

In summary, we synthesized and biologically evaluated Complex I inhibitor **R419** and a series of its derivatives. **R419** and 9 of its derivatives displayed significant inhibitory activity against HepG2 cell. Among them, compound **H20** was the most potent one with $IC_{50}$ value of 2.8±0.4 μM. Flow cytometric analysis revealed that **H20** induced necrotic cell death but not apoptosis. Further mechanism study showed that **H20** caused ATP depletion, AMPK activation and Bcl-2/Bax ratio decrease in HepG2 cells. Enzyme activity assay demonstrated that **H20**, similar to **R419**, had moderate potency of Complex I inhibition. Taken together, **H20** may induce destruction of mitochondrial energy metabolism, at least in part through Complex I-dependent pathway.

## Acknowledgments

Financial support from the National Natural Science Foundation of China (31501182, 81730094, 81473262 and 81573299) is gratefully acknowledged. This project was also supported by the "111 Project" from the Ministry of Education of China, the State Administration of Foreign Expert Affairs of China (No. 111-2-07) and the Fundamental Research Funds for the Central Universities (2632017ZD05).

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## Supplementary Material

Supplementary material is prepared and provided as a separate electronic file.

1. First report on anticancer activity of Complex I inhibitor **R419**
2. Twenty **R419** derivatives were synthesized
3. **H20** is the most potent compound against HepG2 cells
4. **H20** caused cellular ATP depletion and necrosis
5. **H20** displayed inhibitory activity against Complex I