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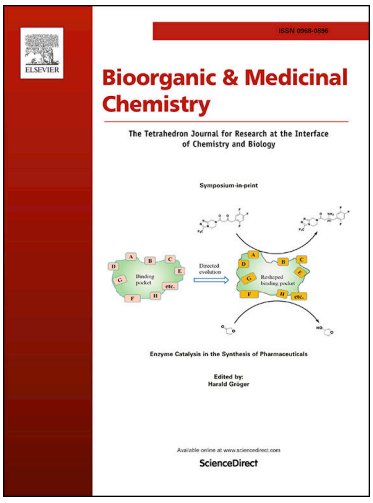

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Identification of small molecule inhibitors of human COQ7

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Key words: COQ7; Ubiquinone; UQ; CoQ; inhibitor; UQ synthesis.

Abstract

Given that the associated clinical manifestations of ubiquinone (UQ, or coenzyme Q) deficiency diseases are highly heterogeneous and complicated, effective new research tools for UQ homeostasis studies are awaited. We set out to develop human COQ7 inhibitors that interfere with UQ synthesis. Systematic structure-activity relationship development starting from a screening hit compound led to the identification of highly potent COQ7 inhibitors that did not disturb physiological cell growth of human normal culture cells. These new COQ7 inhibitors may serve as useful tools for studying the balance between UQ supplementation pathways: *de novo* UQ synthesis and extracellular UQ uptake.

1. Introduction

Ubiquinone (UQ; also known as coenzyme Q) is a redox-active lipid molecule comprised of a benzoquinone and a polyisoprenoid side chain. UQ has important roles in energy metabolism and reactive oxygen species (ROS) homeostasis in cells. UQ functions as an aerobic electron transport mediator from complex I and II to complex III in eukaryotic mitochondria.[1] In the electron transport system in mitochondria, oxidation-reduction reaction mediated by UQ yields intracellular ROS as a major by-product,[2] and simultaneously, the reduced form of UQ regenerates antioxidants, including vitamin E.[1, 3]

Clinically, UQ deficiencies are associated with highly heterogeneous and complicated manifestations. Primary UQ deficiencies are very rare and genetically caused by mutations in genes involved in UQ biosynthesis. These mutations affect multiple organ systems including the central and peripheral nervous systems, kidney, skeletal muscle, heart, and sensory system.[4] Secondary UQ deficiencies caused by defects not directly linked to UQ biosynthesis enzymes are more common than primary UQ deficiencies and are associated with various disorders, including mitochondrial myopathies.[4, 5] The underlying biological mechanisms of attenuated UQ levels in the patients remain largely unknown. A majority of the UQ-deficient patients respond well to oral

supplementation of high-dose UQ_{10}, but in some cases, the clinical response to this therapy is less than satisfactory.[6]

Mammalian cells have two UQ supplementation pathways: endogenous synthesis through the UQ biosynthetic complex in mitochondria,[4, 6, 7] and exogenous UQ uptake.[8, 9] In the UQ synthetic pathway, 4-hydroxybenzoic acid (4-HB), precursor of the benzoquinone, and the isoprenoid tail are enzymatically linked by COQ2, and the resulting molecule undergoes subsequent modifications of the ring moiety.[4] This pathway is regulated in cells by controlling a key enzyme COQ7.[10-12] COQ7 activity is reported to be controlled at the transcription level by NFκB,[13] at the mRNA stabilization level by HuR and hnRNP C1/C2,[14] and at the phosphorylation level by PPTC7.[15] On the other hand, there is almost no report about the regulation of UQ uptake activity, other than that most patients with UQ deficiency respond well to oral UQ_{10} supplementation. A recent report indicates that the scavenger receptor CD36 is responsible for this function.[8] The precise mechanism which regulates these two pathways to achieve UQ homeostasis in human cells is largely unknown, but elucidation of the mechanism is necessary to reveal the pathogenesis of secondary UQ deficiencies. Given a number of questions to be resolved, new effective research tools are needed for studying the biochemical aspects of UQ deficiency syndrome.

In this study, we have identified new inhibitors of COQ7, the key UQ synthetic enzyme.[16, 17] COQ7 associates with COQ9 and forms large UQ biosynthetic complex with other enzymes involved in UQ synthesis on the matrix face of the inner mitochondrial membrane.[18] Human COQ7 is a DMQ mono-oxygenase consisting of 179 amino acids, and its active site is predicted to have a typical di-iron center.[19, 20] COQ7 converts demethoxy-UQ (DMQ) to demethyl-UQ (DMeQ), which is then converted to UQ by COQ3.[21] Thus, cells under COQ7 deficiency are unable to produce UQ, and consequently accumulate DMQ.[22, 23] To date, clioquinol (CQ), 5-chloro-7-indo-8-hydroxyquinoline, is the only reported inhibitor of COQ7 hydroxylase activity.[24] However, CQ is known to form a stable complex with copper (II) ion and thereby exhibits serious cytotoxicity in culture cells. Kawamura et al. studied the cytotoxicity of CQ and reported that inhibition of superoxide dismutase-1 and resulted enhancement of ROS production led the CQ-treated cells to death.[25]

Searches for other COQ7 inhibitors have met with little success, presumably because of the lack of desirable enzymatic assays that can evaluate COQ7 hydroxylase activity. So far, stable COQ7 forming complex with COQ9 has not yet been purified, thus reported recombinant COQ7 preparation has weak hydroxylase activity.[26] To overcome this difficulty, we evaluated COQ7 inhibition activity of test compounds by their effects on UQ synthesis in co-cultured cells.

Compounds we identified in this study revealed that the balance between two UQ supplementation pathways varies widely depending on the cell line.

2. Results and Discussion

2.1. Cell-based screening for hCOQ7 inhibitors

Since the UQ content in cells is known to vary widely among animal tissues,[27] accumulation levels of DMQ_{10} caused by inhibition of UQ synthesis may vary depending on the cell line. Therefore, to select a sufficiently sensitive cell line for our cell-based UQ assay, we tested 5 human cell lines: BxPC3, C3A, PANC-1, PC3, and HeLa. When cultured under normal conditions with no inhibitor, the quinone fractions of these cells contained only UQ_{10} (section 4.3.2, Fig. 3A). After the cells were co-cultured with 10 μM CQ for 1 day, cells are expected to accumulate DMQ_{10} as previously reported (section 4.3.2, Fig. 3B).[24] The amounts of DMQ_{10} and UQ_{10} in each extract were analyzed by using high-pressure liquid chromatography (HPLC), and the proportion of DMQ_{10} content relative to the total quinone content (DMQ_{10} peak height / [UQ_{10} peak height + DMQ_{10} peak height] (%)) was calculated for each extract. The DMQ_{10} content of extracts from C3A and HeLa cells exceeded 20%, whereas extracts from BxPC3, PANC-1, and PC3 cells contained less than 5% DMQ_{10}. These observations suggested that only C3A and HeLa cells

actively synthesize UQ_{10} under this culture condition. When we extend the co-culture with 10 μM CQ to 2 days, the DMQ_{10} contents of the extracts were largely unchanged from those after 1 day co-culture.

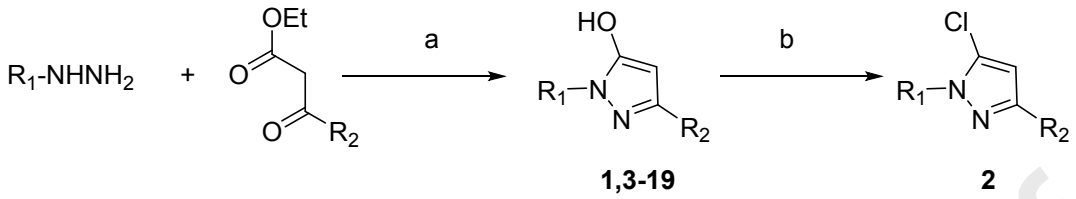
HeLa cells were selected for use in our cell-based screening assay. They were co-cultured for 2 days with each screening compound at 30 μM, cell pellets were prepared from the co-cultures, and their quinone fractions were extracted and analyzed. Among the 141 compounds tested, compound **1** (3-Phenyl-1-(pyridine-2-yl)-1*H*-pyrazol-5-ol) caused remarkable levels of DMQ_{10} accumulation (>30% of total quinone).

Compound **1** was purchased from Labotest, and HeLa cells were co-cultured for 2 days with the compound at 10 μM. The DMQ_{10} content of the resulted extract was 19.3% ± 5.2% (mean ± SD of triplicate results). Thus, we decided to initiate systematic structure-activity development starting from compound **1** for more potent COQ7 inhibitors.

2.2. Development of improved compounds

2.2.1. Synthesis

Scheme 1 shows the synthesis of compounds **1**–**19**. Pyrazole derivatives **1** and **3**–**19** were synthesized by cyclization of the corresponding hydrazine derivatives and β-ketoacetates in EtOH.[28] Chlorination of compound **1** to compound **2** was accomplished by heating in $POCl_3$.

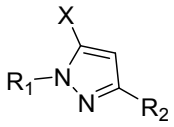


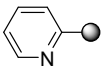
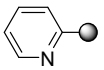
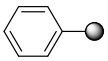
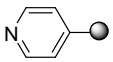
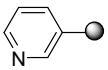
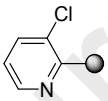
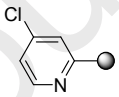
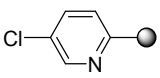
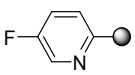
Scheme 1. Reagents and conditions.
a) EtOH, 90 °C; b) $POCl_3$, 110 °C.

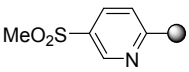
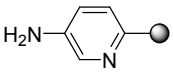
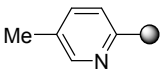
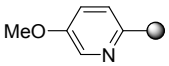
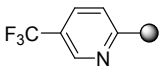
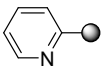
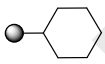
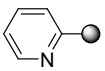
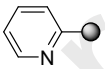
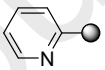
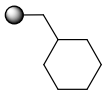
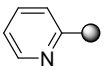
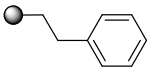
2.2.2. Structure-activity relationships of the hCOQ7 inhibitors

COQ7 inhibition activity and cytotoxic effect of the synthetic compounds were determined (Table 1). Inhibition of COQ7 activity was evaluated by the cell-based quinone analysis of extracts from HeLa cells co-cultured with each test compound at 10 μM for 2 days, and DMQ_{10} and UQ_{10} contents in each culture well (ng/well), and calculated DMQ_{10} content (%) were determined. The effect of each compound on COQ7 activity was evaluated as DMQ_{10} content (%) as discussed before (see section 2.1.), because total quinone content (ng) was affected by the number of cells harvested. The cytotoxic effect of the compounds was evaluated by growth of human normal cell line WI-38 co-cultured with various concentrations (0-30 μM) of tested compounds for 4 days. The concentration of each compound that caused 50% cell growth inhibition (GI50) was determined and listed in Table 1.

Table 1. UQ_{10} synthesis-inhibiting activity of pyrazole derivatives.



Compound No.	R_1	R_2	X	DMQ_{10}* (ng/well)	UQ_{10}* (ng/well)	DMQ_{10} content* (%)	WI-38 GI50 (μM)
1		Ph	OH	1.3	17.1	7.1	20.8
2		Ph	Cl	0	35.7	0	>30
3		Ph	OH	0	28.5	0	>30
4		Ph	OH	0	33.7	0	>30
5		Ph	OH	0	23.7	0	>30
6		Ph	OH	0	20.7	0	>30
7		Ph	OH	0	22.7	0	>30
8		Ph	OH	13.2	12.2	52.0	19.0
9		Ph	OH	1.8	14.8	10.8	28.9

10		Ph	OH	2.2	17.8	11.0	>30
11		Ph	OH	2.6	23.4	10.0	>30
12		Ph	OH	7.3	15.4	32.2	26.3
13		Ph	OH	0	6.7	0	25.4
14		Ph	OH	9.2	9.4	49.5	9.4
15			OH	5.5	5.9	20.7	9.1
16			OH	5.8	12.1	32.4	12.7
17			OH	0	24.4	0	25.0
18			OH	6.6	8.8	42.9	17.5
19			OH	9.3	8.8	51.4	9.6

*: mean value of duplicate results.

No accumulation of DMQ_{10} was observed in cells co-cultured with compound **2**. Therefore,

replacement of the hydroxyl group in compound **1** with chlorine (**2**) abolished the COQ7 inhibition activity, suggesting that the hydroxyl group is essential for the interaction with COQ7. In addition, compounds **3-5** failed to cause DMQ_{10} accumulation, suggesting that the 2-pyridyl structure is critically necessary for the activity (Table 1).

Next, the contribution of the substituent on the 2-pyridyl group of compound **1** to DMQ_{10} accumulation was studied. Introduction of the 3-chloro and 4-chloro groups (**6** and **7**) resulted in decreased activity. In contrast, the 5-chloro analog (**8**) was more potent than **1**, suggesting that introduction of an appropriate substituent at the 5-position may lead to increased COQ7 inhibition. This finding prompted us to study the effects of other functional groups at the 5-position. In our limited examples, the order of DMQ_{10} contents (%) in HeLa cells was as follows: chlorine (**8**) > trifluoromethyl (**14**) > methyl (**12**) > sulfonyl methyl (**10)** > fluorine (**9**) > amine (**11**) > methoxy (**13**).

When alkyl groups with various sizes (compounds **15-18**) and an aralkyl group (compound **19**) were introduced to the R2 group, it appeared that bulky groups (compounds **15**, **16**, **18**, and **19**) gave more desirable DMQ_{10} accumulation than the phenyl group (compound **1**). However, these compounds showed increased cytotoxicity.

Considering the balance between the desirable COQ7 inhibitory activity and the undesirable

cytotoxicity against the human normal cell line indicated by the GI50 values, compounds **8** and **12** appeared to have the most favorable characteristics (Table 1).

2.3. Characterization of the COQ7 inhibitors

2.3.1. Effects of the COQ7 inhibitors on human culture cells

Since compounds **8** and **12** had less growth inhibition against human normal culture cells (Table 1), their effects on human cancer-derived cell lines (C3A, HeLa, PANC-1, and PC-3) were investigated. The GI50 values of the two compounds against the four cell lines were determined, and we found that all the values were higher than 12 μM, except the value of compound **8** against C3A cells (discussed later). When co-cultured with CQ, a known inhibitor of COQ7, C3A and HeLa cells highly accumulated DMQ_{10}, whereas PANC-1 and PC-3 cells did not (see section 2.1.). These observations demonstrated the weak toxicity of compounds **8** and **12** against these cell lines, regardless of the levels of accumulated DMQ_{10}.

Since weak growth inhibition of C3A cells was observed with compounds **8** (GI50, 9.0 ± 1.1 μM) and **12** (GI50, 18.5 ± 1.2 μM)), biological damage induced by these COQ7 inhibitors were studied using C3A cells. The first likely target of inhibitor-induced damage was electron mediation in the respiratory chain, but we thought that the accumulated DMQ_{10} can function instead of UQ_{10} in the respiration and thus is nontoxic for human cells, as it is known for *C. elegance*.[22] To confirm

this point, we determined the GI50 values of the compounds against C3A cells cultured in medium containing 10 mM 2-deoxyglucose (2-DG), which inhibits glycolysis.[29-32] Under these conditions, cells utilize aerobic respiration only for their primary means of energy production. Thus consequently C3A cells exhibited growth retardation (generation time extended approximately 2-fold). However, the GI50 values of the COQ7 inhibitors stayed largely unchanged (GI50 values: compound **8**, 6.1 ± 0.32 μM; compound **12**, 20.2 ± 1.99 μM), suggesting that the accumulated DMQ_{10} does not inhibit cellular aerobic respiration in our assay system. It was in good agreement with previous studies by Wang *et al.*, in which mitochondrial preparations from coq7 KO mice required only mild UQ addition for the electron transport.[23, 33]

The next likely target of the inhibitor-induced damage is the anti-oxidant defense systems. Therefore, we added 0.3, 1, and 3 mM $CoCl_2$ to C3A cultures to increase the generation of cellular ROS.[34, 35] Under these conditions, cell viabilities with 0.3, 1, and 3 mM $CoCl_2$ at 24 hr were decreased to 78%, 51%, and 33%, respectively. Simultaneous addition of compound **8** or **12** at 10 μM was tested, and the addition did not increase the amount of cell death to meaningful extents. Recent reports on transgenic mice study[7] and pathogenesis of UQ deficiency[4, 6] indicated that severe UQ deficiency, *per se*, does not increase the levels of oxidative stress. Together, these

findings suggested that our COQ7 inhibitors do not limit C3A cell survival through inhibiting aerobic respiration or increasing oxidative stress.

HeLa cells were co-cultured for 2 days with CQ, compounds **8** and **12** at various concentrations, and their quinone fractions were analyzed (Fig.1). In the case of CQ treatment, DMQ accumulation was observed with 5 μM addition, but it decreased with 20 μM addition due to the severe cytotoxicity of CQ. On the other hand, extracts of cells treated with higher concentrations of the new compounds showed increased DMQ_{10} contents (%). Saturation of DMQ_{10} content occurred at approximately 60% of the total quinone content, and the addition of compound **8** at 10 μM was more effective to increase the DMQ_{10} content as compared with when compound **12** was employed. These results showed usefulness of our new inhibitors to block UQ synthesis without disturbing the condition of culture cells.

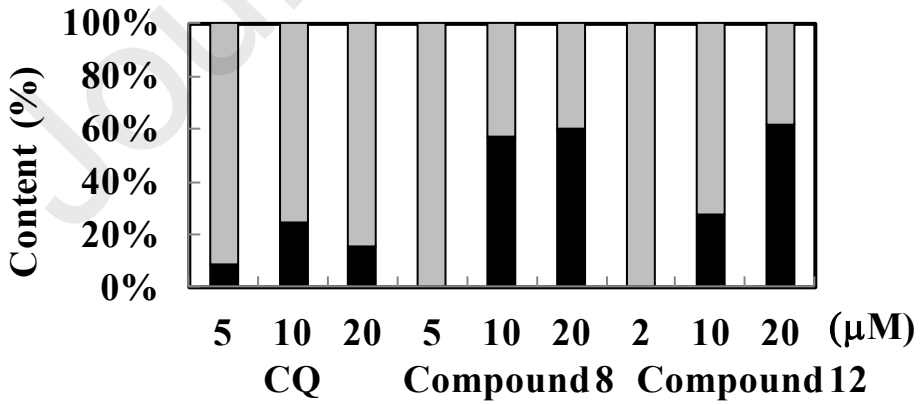


Fig. 1 Analysis of UQ_{10} and DMQ_{10} in HeLa cells treated with COQ7 inhibitors.
After 2 days of co-culture with CQ (left), compound **8** (middle), or **12** (right) at the indicated concentrations, HeLa cells were harvested and their UQ_{10} and DMQ_{10} contents (%) were analyzed. The gray part of each bar indicates the amount of UQ_{10}, and the black part indicates that of DMQ_{10}. The mean values of duplicate results are shown.

2.3.2. UQ supplementation pathways in human culture cells

The quinone content of various cell lines cultured for 1 day with the inhibitors at 10 μM was analyzed (Fig. 2). To our surprise, substantial DMQ_{10} accumulation occurred in both PANC-1 cells (11.4% by compound **8**, and 8.7% by compound **12**) and PC3 cells (27.6% by compound **8**, and 14.0% by compound **12**). In contrast, DMQ_{10} accumulations did not apparently occur in either PANC-1 or PC3 cells treated with CQ (section 2.1). These observations indicated that treatment with our non-toxic COQ7 inhibitors revealed that culture cells of all the human strains tested actively synthesize UQ_{10} through COQ7.

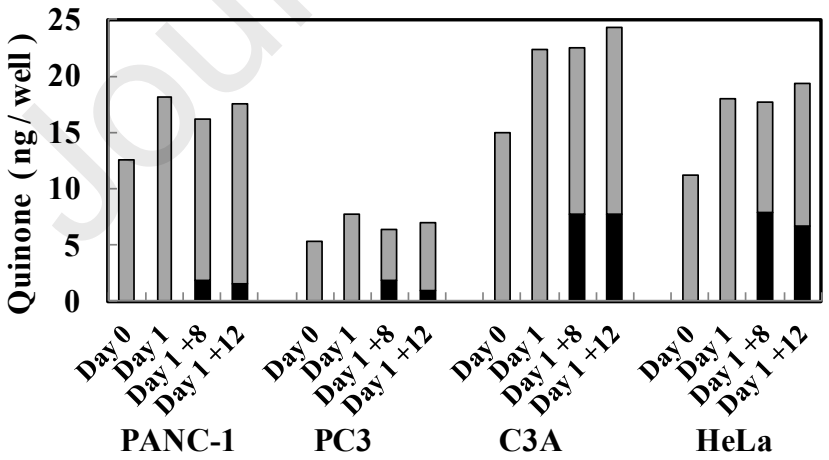


Fig. 2 COQ7 inhibitors reveal that the balance between UQ supplementation pathways varies depending on the cell line.
PANC-1, PC3, C3A, and HeLa cells were co-cultured for 1 day with compound **8** or **12** at 10 μM, and the resulting quinone contents (ng) were determined. Each bar indicates the total amount of UQ_{10} and DMQ_{10} in the untreated control culture (Day 0), control culture treated with DMSO (Day 1), and the cultures treated with compound **8** (Day 1 +**8**) or **12** (Day 1 +**12**). The gray part of each bar represents the amount of UQ_{10}, and the black part represents the amount of DMQ_{10}. The mean values of duplicate results are shown.

The increase of the total amount of UQ_{10} and DMQ_{10} during 1 day (ng per culture well) was studied (Fig. 2). That in the untreated C3A culture was 7.4 ng, and in the HeLa culture was 6.8 ng. On the contrary, the amount of accumulated DMQ_{10} in C3A culture treated with compound **8** for 1 day was 7.8 ng, and that in HeLa culture was 7.9 ng. These results suggested that UQ supplementation in C3A and HeLa cells was mainly due to the *de novo* UQ synthesis through COQ7. In contrast, the total amount of quinones increased by 5.5 ng in PANC-1 culture for 1 day, but the DMQ_{10} accumulation was less than 2 ng. These findings suggested that uptake of extracellular UQ_{10} primarily accounts for UQ supplementation in PANC-1 cells. Therefore, our COQ7 inhibitors revealed that the balance between UQ supplementation pathways, *de novo* UQ_{10} synthesis, and uptake of extracellular UQ_{10}, varies widely depending on the cell line.

Thelin *et al.* reported that the half-life of UQ_9 in rat varied between 49 and 125 hours, depending on the tissue evaluated.[27] According to the UQ half-life, 10% to 30% of UQ_{10} present

in cells might be degraded during 1 day culture. It means that the actual amount of newly synthesized UQ_{10} in the "Day 1" sample is somewhat larger than that calculated from the difference between that in "Day 1" sample and in "Day 0" sample (Fig. 2). Therefore, our inhibitors might not completely inhibit UQ_{10} synthesis in these cells, yet as shown in Fig. 2, our inhibitors are useful tools for analyzing the balance between the UQ supplementation pathways. The information will contribute to the biochemical understanding of the pathogenesis of secondary UQ deficiency.

3. Conclusions

We have identified a series of new COQ7 inhibitors that interfere with cellular UQ synthesis, and we used these compounds to show that the balance between the UQ supplementation pathways varies widely depending on the cell line studied. In particular, compounds **8** and **12** may serve as effective tools for studying the UQ supplementation pathways, and contribute to the elucidation of the mechanism that causes secondary UQ deficiency.

4. Materials and Methods

4.1. Screening compounds

A total of 141 compounds were obtained from the Drug Discovery Initiative, at the University

of Tokyo, Japan (http://www.ddi.u-tokyo.ac.jp/). CQ was purchased from Fluka.

4.2. Synthetic compounds

4.2.1. General

All reagents and solvents were obtained from commercial sources and used as received. ^{1}H NMR spectra were recorded with tetramethylsilane (TMS) as the internal standard by using a 270 MHz spectrometer (JNM-Ex, JEOL). Automated column chromatographic separations were performed on a flash chromatograph (Biotage ZIP Si, Biotage Inc., and Hi Flash amino, Maruzen Inc.). LC-MS analysis was performed by Aquity Ultra Performance LC (Waters) using an Aquity UPLC BEH C18 column (2.1 mm x 50 mm, Waters). The column temperature was 40 °C, run time was 2 min, flow rate was 0.6 ml/min, and the eluting solvent was a mixture of acetonitrile and water containing 0.1% trifluoroacetic acid with a gradient of 10%-90%. The mass spectrometry data were acquired on a SQD2 quadrupole mass spectrometer (Waters). The purity of all synthesized compounds exceeded 95%.

*4.2.2. 3-Phenyl-1-(pyridin-2-yl)-1H-pyrazol-5-ol (**1**)*

Compound **1** was synthesized as reported before.[36] ^{1}H NMR (270 MHz, $CDCl_3$) δ 12.81(1H, s), 8.27-8.30(1H, m), 8.04-8.08 (1H, m), 7.84-9.94 (3H, m), 7.33-7.46 (3H, m), 7.15-7.20(1H, m), 5.95 (1H, s). LC-MS (ESI): *m/z* 316 $[M+H]^+$

*4.2.3. 2-(5-Chloro-3-phenyl-1H-pyrazol-1-yl)pyridine (**2**)*

Compound **1** (50 mg, 0.211 mmol) was heated in $POCl_3$ (350 µl) for 3 h. After cooling to room temperature, the reaction mixture was carefully poured into ice water, extracted with dichloromethane, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The residue was purified by column chromatography over silica gel (hexane/EtOAc) to afford **2** (33 mg, 61%) as a white solid. ^{1}H NMR (270 MHz, $CDCl_3$) δ 8.58-8.60 (1H, m), 7.80-7.93 (4H, m), 7.30-7.46 (4H, m), 6.75 (1H, s) LC-MS (ESI): *m/z* 256 $[M+H]^+$

*4.2.4. 1,3-Diphenyl-1H-pyrazol-5-ol (**3**)*

To a solution of phenylhydrazine (182 µM, 200 mg, 1.849 mmole), ethyl benzoylacetate (382.9 µl, 426.5 mg, 2.219 mmole) in EtOH (0.5 ml) was added and stirred at 90 °C overnight in a sealed tube. After cooling to room temperature, the solid obtained was filtered, washed with EtOH, and dried to afford **3** (112 mg, 26% yield) as a yellow solid. ^{1}H NMR (270 MHz, $CDCl_3$) δ 7.95-8.00 (2H, m), 7.75-7.80 (2H, m), 7.40-7.49 (5H, m), 7.19-7.26 (1H, m), 3.87 (2H, s). LC-MS (ESI): *m/z* 237 $[M+H]^+$

4.2.5. 3-Phenyl-1-(pyridin-4-yl)-1H-pyrazol-5-ol (***4***)

Compounds **4-19** were prepared by using a similar procedure as that for compound **3**. ^{1}H NMR (270 MHz, DMSO-d_6) δ 8.59 (2H, d, J = 6.5 Hz), 7.98 (2H, d, *J* = 6.5 Hz), 7.84-7.87 (2H,

m), 7.34-7.47 (3H, m), 5.96 (1H, s). LC-MS (ESI): *m/z* 238 $[M+H]^+$

*4.2.6. 3-Phenyl-1-(pyridin-3-yl)-1H-pyrazol-5-ol (**5**)*

^{1}H NMR (270 MHz, DMSO-d_6) δ 9.08 (1H, s), 8.48 (1H, d, J = 4.8 Hz), 8.22 (1H, d, J = 8.1 Hz), 7.84 (2H, d, J = 7.6 Hz), 7.35-7.55 (4H, m), 6.04 (1H, s). LC-MS (ESI): *m/z* 238 $[M+H]^+$

*4.2.7. 1-(3-Chloropyridin-2-yl)-3-phenyl-1H-pyrazol-5-ol (**6**)*

^{1}H NMR (270 MHz, $CDCl_3$) δ 11.37 (1H, s), 7.95-7.87 (3H, m), 7.81-7.87 (3H, m), 7.34-7.46 (3H, m), 7.16-7.20 (1H, m) 5.98 (1H, s). LC-MS (ESI): *m/z* 272 $[M+H]^+$

4.2.8. 1-(4-Chloropyridin-2-yl)-3-phenyl-1H-pyrazol-5-ol (7)

^{1}H NMR (270 MHz, $CDCl_3$) δ 8.38 (1H, d, J = 5.7Hz), 8.03 (1H, d, J = 1.6 Hz), 7.97-8.00 (1H, m), 7.78-7.82 (2H, m), 7.46-7.55 (3H, m), 3.91 (2H, s). LC-MS (ESI): *m/z* 272 $[M+H]^+$.

*4.2.9. 1-(5-Chloropyridin-2-yl)-3-phenyl-1H-pyrazol-5-ol (**8**)*

^{1}H NMR (270 MHz, $CDCl_3$) δ 11.94 (1H, s), 8.26-8.27 (1H, m), 8.02-8.05 (1H, m), 7.79-7.89 (3H, m), 7.33-7.48 (3H, m), 5.96 (1H, s). LC-MS (ESI): *m/z* 272 $[M+H]^+$.

4.2.10. 1-(5-Fluoropyridin-2-yl)-3-phenyl-1H-pyrazol-5-ol (**9**)

^{1}H NMR (270 MHz, $CDCl_3$) δ 11.93 (1H, s), 8.17 (1H, d, J = 2.7 Hz), 8.07-8.12 (1H, m), 7.81-7.87 (2H, m), 7.63-7.71 (1H, m), 7.33-7.48 (3H, m), 5.96 (1H, s). LC-MS (ESI): *m/z* 256 $[M+H]^+$.

*4.2.11. 1-(5-(Methylsulfonyl)pyridin-2-yl)-3-phenyl-1H-pyrazol-5-ol (**10**)*

^{1}H NMR (270 MHz, $CDCl_3$) δ 11.90 (1H, s), 8.86 (1H, d, *J* = 2.2 Hz), 8.35-8.39 (1H, m), 8.23 (1H, d, *J* = 8.9 Hz), 7.85-7.89 (2H, m), 7.37-7.49 (3H, m), 6.01 (1H, s), 3.169 (3H, s). LC-MS (ESI): *m/z* 316 $[M+H]^+$.

*4.2.12. 1-(5-Aminopyridin-2-yl)-3-phenyl-1H-pyrazol-5-ol (**11**)*

^{1}H NMR (270 MHz, $CDCl_3$) δ 12.51 (1H, s), 7.83-7.89 (3H, m), 7.73 (1H, d, *J* = 2.4 Hz), 7.30-7.44 (1H, m), 7.22-7.25 (1H, t), 5.92 (1H, s), 3.72 (2H, s). LC-MS (ESI): *m/z* 253 $[M+H]^+$.

4.2.13. 1-(5-Methylpyridin-2-yl)-3-phenyl-1H-pyrazol-5-ol (***12***)

^{1}H NMR (270 MHz, $CDCl_3$) δ 12.84 (1H, s), 8.10 (1H, d, *J* = 2.2 Hz), 7.95 (1H, d, *J* = 8.4 Hz), 7.84-7.88 (1H, m), 7.70-7.74 (1H, m), 7.32-7.47 (4H, m), 5.94 (1H, s), 2.38 (3H, s). LC-MS (ESI): *m/z* 252 $[M+H]^+$.

*4.2.14. 1-(5-Methoxypyridin-2-yl)-3-phenyl-1H-pyrazol-5-ol (**13**)*

^{1}H NMR (270 MHz, $CDCl_3$) δ 12.39 (1H, s), 7.99-8.02 (1H, m), 7.94-7.96 (1H, m), 7.83-7.87 (2H, m), 7.31-7.50 (4H, m), 5.94 (1H, s), 3.90 (3H, s). LC-MS (ESI): *m/z* 268 $[M+H]^+$.

*4.2.15. 3-Phenyl-1-(5-(trifluoromethyl)pyridin-2-yl)-1H-pyrazol-5-ol (**14**)*

^{1}H NMR (270 MHz $CDCl_3$) δ 12.04 (1H, s), 8.59 (1H, s), 8.09-8.59 (2H, m), 7.84-7.88 (2H, m), 7.36-7.50 (3H, m), 5.99 (1H, s). LC-MS (ESI): *m/z* 306 $[M+H]^+$.

*4.2.16. 3-Cyclohexyl-1-(pyridin-2-yl)-1H-pyrazol-5-ol (**15**)*

^{1}H NMR (270 MHz, $CDCl_3$) δ 12.70 (1H, s), 8.23 (1H, d, *J* = 5.1 Hz), 7.80-7.90 (2H, m), 7.08-7.13 (1H, m), 5.44 (1H, s), 2.54-2.61 (1H, m), 1.71-1.99 (1H, m), 1.27-1.51 (5H, m). LC-MS (ESI): *m/z* 244 $[M+H]^+$.

*4.2.17. 3-Cyclopentyl-1-(pyridin-2-yl)-1H-pyrazol-5-ol (**16**)*

^{1}H NMR (270 MHz, $CDCl_3$) δ 12.74 (1H, s), 8.23-8.25 (1H, m), 7.80-7.91 (2H, m), 7.08-7.13 (1H, m), 5.44 (1H, s), 3.00-3.06 (1H, m), 2.00-2.06 (2H, m), 1.62-1.81 (6H, m). LC-MS (ESI): *m/z* 230 $[M+H]^+$.

*4.2.18. 3-Cyclobutyl-1-(pyridin-2-yl)-1H-pyrazol-5-ol (**17**)*

^{1}H NMR (270 MHz, $CDCl_3$) δ12.74 (1H, s), 8.22-8.25 (1H, m), 7.80-7.91 (2H, m), 7.09-7.17 (1H, m), 5.53 (1H, s), 3.46-3.53 (1H, m), 2.17-2.37 (4H, m), 1.89-2.08 (2H, m). LC-MS (ESI): *m/z* 216 $[M+H]^+$.

*4.2.19. 3-(Cyclohexylmethyl)-1-(pyridin-2-yl)-1H-pyrazol-5-ol (**18**)*

^{1}H NMR (270 MHz, $CDCl_3$) δ12.74 (1H, s), 8.22-8.25 (1H, m), 7.80-7.91 (2H, m), 7.09-7.17 (1H, m), 5.53 (1H, s), 3.46-3.53 (1H, m), 2.17-2.37 (overlapped by water signal, 13H, m), 1.89-2.08 (2H, m). LC-MS (ESI): *m/z* 258 $[M+H]^+$.

*4.2.20. 3-Phenethyl-1-(pyridin-2-yl)-1H-pyrazol-5-ol (**19**)*

^{1}H NMR (270 MHz, $CDCl_3$) δ 12.70 (1H, s), 8.21-8.26 (1H, m), 7.81-7.91 (2H, m), 7.27-7.33 (4H, m), 7.07-7.23 (2H, m), 5.43 (1H, s), 2.86-3.04 (4H, m). LC-MS (ESI): *m/z* 266 $[M+H]^+$.

4.3. Cell culture and cell-based assays

4.3.1. Cell culture

Human pancreas adenocarcinoma BxPC-3 cells, human hepatocellular carcinoma derived C3A cells, human pancreas epithelioid carcinoma PANC-1 cells, and human prostate adenocarcinoma PC-3 cells, were purchased from American Type Culture Collection. Human cervix epidermoid carcinoma HeLa cells and human normal fibroblast WI-38 cells were obtained from the RIKEN BioResource Center. HeLa, WI-38 and PANC-1 cells were cultured in Dulbecco's Modified Eagle's Medium (GIBCO, Carlsbad, CA, USA), containing 10% fetal bovine serum (FBS) and penicillin (5,000 U/ml)–streptomycin (5 mg/ml), at 37 °C in a 5% CO_2 atmosphere. BxPC-3 cells were cultured in RPMI-1640 Medium (GIBCO), C3A cells were in Eagle's Minimum Essential Medium (GIBCO), and PC-3 cells were in F-12K Medium (GIBCO), containing 10% FBS and the antibiotics. The cells were pre-cultured overnight and then subsequently cultured with each compound or 0.5% DMSO (control).

The effects of compounds on the growth of the human cells were evaluated by using the CellTiter-Glo 2.0 assay (Promega). Various concentrations of the test compounds were added to

cells pre-cultured for 24 hr. The cells were cultured for further 3 days (PANC-1, PC-3, C3A, and HeLa), or 4 days (WI-38), and the numbers of viable cells were determined. GI50 values, reported as the mean values of triplicate results, were determined through linear interpolation from concentration-response curves. To inhibit glycolysis 2-DG was added to the culture medium at the concentration of 10 mM.[29]

4.3.2. Analysis of quinones

Cells (2 or 4 x 10^5 cells per well) were inoculated and cultured overnight, after which test compounds were added and co-cultured for further 24 or 48 hr. Control samples were co-cultured with 0.5%-1.0% DMSO. In most cases, we prepared 2 culture wells for each experimental condition.

Harvested cells were suspended in phosphate-buffered saline (PBS) containing 5% $K_3Fe(CN)_6$. After addition of 0.12 μg UQ_9 as the internal standard, quinone fractions were extracted from the cell suspension by vigorous mixing in 7 volumes of ethanol/*n*-hexane (2:5, v/v) solution for 10 min, and then centrifuged at 9,100 x *g* for 5 min at room temperature. The supernatants were pooled and dried in an evaporation device, and the residues were dissolved in 30 μl methanol and analyzed by HPLC with a UV detector (275 nm) (UV-2075, JASCO, Tokyo, Japan). A 10 μl aliquot of the quinone fraction was injected to a reverse-phase HPLC column (CAPCELL PAK C18IF S2, 2 μm,

2.0 x 50 mm, OSAKA SODA, Osaka, Japan) and eluted under isocratic conditions (0.15 ml/min, 30 °C) by using diisopropyl ether/methanol (1:9, v/v), as described previously.[22, 37] The spectral characteristics of each quinone were monitored by using a photodiode array UV-visible detector MD-2018 (JASCO).

4.3.3. Cell-based evaluation for hCOQ7 inhibitors

To select COQ7 inhibitors from test compounds, we measured the accumulated amounts of the precursor molecule, DMQ_{10}, in co-cultured cells. A specific peak detected by our HPLC device was assigned as the DMQ_{10} peak in the following manner. An overnight culture of HeLa cells was further co-cultured with 1% DMSO (negative control) or 10 μM CQ (positive control) for 1 day, and then cells were harvested, and their quinone fractions were extracted to measure the cellular contents of DMQ_{10} and UQ_{10}. The extract of negative control cells yielded two peaks, at 8.1 and 11.1 min (Fig. 3A), which we assigned to UQ_9 and UQ_{10}, respectively. UQ_{10} was the endogenous UQ in human cells, and UQ_9 was the internal standard that we added to the cell pellets. Extracts of CQ-treated cells yielded a third peak, at 10.2 min (Fig. 3B), corresponding to DMQ_{10} as reported previously.[24] The treatment with CQ inhibited COQ7 that catalyzes DMQ_{10} to UQ_{10}, and thus the extract of treated cells contained DMQ_{10} fraction.

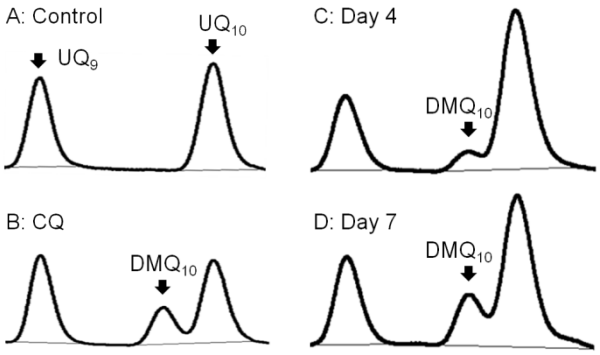


Fig. 3 Representative chromatograms of quinone fractions extracted from human cells. Quinone fractions were extracted from human HeLa cells co-cultured for 1 day with 1% DMSO (A) or 10 μM CQ (B). HeLa cells transfected with COQ7-siRNA were harvested and tested at 4 days (C) and 7 days (D) after the first transfection.

To confirm the assignment of the detected HPLC peak at 10.2 min to DMQ_{10} that accumulated due to COQ7 inhibition, we analyzed the ubiquinone extract from cells with decreased COQ7 levels. HeLa cells were transfected with small interfering RNA (siRNA) to knock down COQ7 protein as reported before.[38] The siRNA targeting human COQ7 (5'-AGUCGGGCAGCUGUGGAUCGAAUAA-3') and control siRNA (Stealth RNAi siRNA Negative Control Med GC Duolex #2) were purchased from Invitrogen. In short, 50 nM siRNA and Lipofectamine RNAiMAX transfection reagent (Invitrogen) diluted in Opti-MEM media (Invitrogen) were incubated for 20 min to prepare the transfection complexes, and HeLa cells were transfected with the transfection complexes according to the manufacturer's instructions.

After 3 days of culture, the amount of COQ7 in the transfected cells was examined by western

blotting using primary antibody (anti-hCOQ7 (F-9), Santa Cruz), and its elimination was confirmed. The ubiquinone fractions extracted from the treated cells after 4 and 7 days showed increase of the HPLC peaks at 10.2 min, which indicated that the peak at 10.2 min corresponds to DMQ_{10} that accumulated due to the insufficiency of COQ7 (Figs. 3C and D). Therefore, we confirmed that the cell-based measurement of the DMQ_{10} amount was available to evaluate COQ7 activity.

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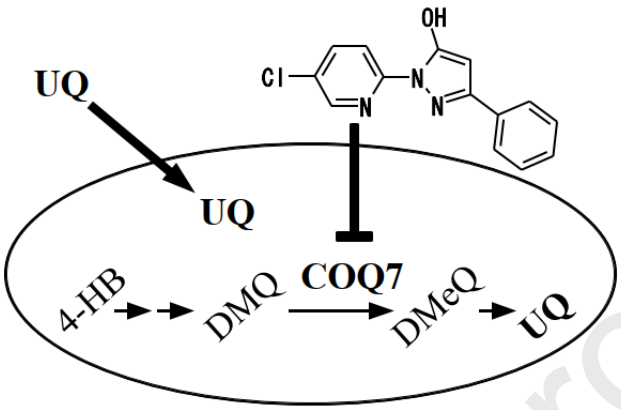
Declaration of interests

✓The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Akiko Tanaka

GA



UQ (CoQ) supplementation in human cells

Highlights

The pathogenesis of ubiquinone (UQ) deficiency syndrome is largely unknown.
We have developed human COQ7 inhibitors that interfere with UQ synthesis.
The highly potent compounds do not disturb growth of human normal culture cells.
The inhibitors will be useful for studying the UQ supplementation pathways.