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Synthesis of 8-hydroxy-2-iminochromene derivatives as selective and potent inhibitors of human carbonyl reductase 1

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Human carbonyl reductase 1 (CBR1), a member of the short-chain dehydrogenase/reductase superfamily, reduces anthracycline anticancer drugs to their less potent anticancer C-13 hydroxy metabolites, which are linked with pathogenesis of cardiotoxicity, a side effect of the drugs. CBR1 inhibitors are thought to be promising agents for adjuvant therapy with twofold beneficial effect in prolonging the anticancer efficacy of the anthracyclines while decreasing cardiotoxicity. In order to search for new potential inhibitors of CBR1, we synthesized a series of des-methoxyphenyl derivatives of (*Z*)-2-(4-methoxyphenylimino)-7-hydroxy-*N*-(pyridin-2-yl)-2*H*-chromene-3-carboxamide (**1**) that was developed previously as a potent inhibitor of aldo-keto reductase (AKR) 1B10 and AKR1B1. Among the newly synthesized inhibitors, 8-hydroxy-2-imino-2*H*-chromene-3-carboxylic acid (2-chlorophenyl)amide (**13h**) was the most potent competitive inhibitor of CBR1, showing a K_i value of 15 nM. **13h** also showed high selectivity to CBR1 over its isozyme CBR3 and other enzymes with CBR activity (AKR1B1, AKR1B10, AKR1C1, AKR1C2, AKR1C4, DXCR and DHRS4). Furthermore, **13h** inhibited the cellular metabolism by CBR1 at its concentration of 4 μM. The structure-activity relationship of the derivatives, site-directed mutagenesis of putative binding residues (Met141 and Trp229) and molecular docking of **13h** in CBR1 revealed that the interactions of **13h** with the substrate-binding residues (Ser139, Met141, Tyr193 and Trp229) are important for the tight binding.

1. Introduction

Carbonyl reductase (CBR, EC 1.1.1.184) catalyzes the NADPH-linked reduction of various aldehydes, ketones and quinones to their corresponding alcohols.[1-3] The enzyme exists in two forms in both the cytosol (CBR1and CBR3) and mitochondria (CBR2 and CBR4) of mammalian cells, and the four CBRs belong to the short-chain dehydrogenase/reductase (SDR) family. Among them, the gene for CBR2 is not present in the human genome and human CBR4 is reported to act as a reductase for quinones and 3-ketoacyl-acyl carrier protein[4,5] Human CBR1 and CBR3 are monomeric proteins sharing 72% amino acid sequence identity, but are clearly different in their enzymatic properties.[3,6,7] CBR1 reduces a wide spectrum of biologically active carbonyl compounds, while CBR3 exhibits limited carbonyl reductase activity. Since homozygous null (*Cbr1* -/-) mice are fetal lethal,[8] CBR1 has been suggested to play pivotal roles in metabolism of endogenous carbonyl compounds. Such endogenous

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substrates of the enzyme have been reported to be isatin,[3,9] prostaglandins,[10] 6-pyruvoyltetrahydropterin,[11] *S*-nitrosoglutathione[12] and lipid peroxidation-derived aldehydes.[2,13,14] However, these substrates are also reduced or metabolized by several aldo-keto reductases (AKRs)[15-17] and other enzymes,[18] and the physiological roles of CBR1 are still not fully elucidated.

CBR1 also reduces a wide variety of xenobiotic carbonyl compounds into their corresponding alcohols, which are easier to be conjugated and eliminated.[1-3]. Thus, the enzyme plays a major role in the phase I metabolism of xenobiotic compounds including drugs, but its carbonyl reduction of anthracycline drugs, doxorubicin and daunorubicin, into their 13-alcohol metabolites, leads to unfavorable bioactivation. The anthracyclines are highly effective anticancer drugs for the treatment of many cancers such as leukemia, soft tissue sarcomas and breast cancer, but their dosages are strictly limited due to increased risk for congestive heart failure depending on the cumulative dose.[19] The pathogenesis of anthracycline-related cardiotoxicity has been linked to the synthesis of anthracycline C-13 alcohol metabolites by CBR1.[19,20] The involvement of this enzyme in the development of chemoresistance and cardiotoxicity during therapy with doxorubicin is also supported by experiments in heterozygous null (*Cbr1* +/-) mice [8] and transgenic mice overexpressing CBR1.[21] Therefore, inhibitors for human CBR1 are thought to be promising agents for adjuvant therapy with twofold beneficial effect in improving the therapeutic response to the anthracyclines while reducing the cardiotoxic side effects in patients undergoing chemotherapy. In addition, selective inhibitors of CBR1 may be useful in elucidating the physiological roles of the enzyme and its contribution to metabolism of newly developed carbonyl-containing drugs.

Human CBR1 is reported to be inhibited by several synthetic and natural compounds (Fig. 1). Representative synthetic inhibitors are ethacrynic acid, indomethacin,[10] 3-(1-*tert*-butyl-4-amino-1*H*-pyrazolo[3,4-d]pyrimidin-3-yl)phenol (Hydroxy-PP),[22] triclosan and zearalenone analogues,[23] of which a zearalenone analogue shows the most potent inhibition (IC_{50} 0.21 μM). The natural inhibitors are curcumin[24] and flavonoids [23,25-27] of which a flavonoid luteolin most potently inhibits CBR1 (IC_{50} 0.095 μM).[27] However, the inhibitory selectivity of the above synthetic CBR1 inhibitors has not been studied, and curcumin and flavonoids are reported to inhibit other enzymes such as human aldose reductase (AKR1B1), aldose reductase-like protein (AKR1B10)[28,29] and/or dehydrogenase/reductase SDR family member 4 (DHRS4).[30]

The aim of this study is to find novel and potent inhibitors that are selective to human CBR1. We previously discovered (*Z*)-2-(4-methoxyphenylimino)-7-hydroxy-*N*- (pyridin-2-yl)-2*H*-chromene-3-carboxamide (**1**) as a potent competitive inhibitor of AKR1B10 and AKR1B1,[31] and subsequently elucidated the structure-activity relationship by synthesis of **1**-derivatives.[32] As a preliminary study to search structurally new CBR1 inhibitors, we examined the inhibitory activities of these chromene derivatives, and found that a des-methoxyphenyl derivative of **1**, 2-(imino)-8-hydroxy-*N*-(pyridin-2-yl)-2*H*-chromene-3-carboxamide (**9**), inhibits CBR1, but not AKR1B10 and AKR1B1. To obtain more potent and selective CBR1 inhibitors, derivatives of **9** were synthesized, and evaluated for their inhibitory activity and selectivity towards CBR1 by comparing their effects on human CBR3, DHRS4 and dicarbonyl/L-xylulose reductase (DCXR)[33] that are members of the SDR superfamily with CBR activity. Since compound **13h** with 2-chlorophenyl moiety instead of the 2-pyridyl moiety of **9** was found to be the most potent competitive inhibitor of CBR1 (IC_{50} 34 nM), docking simulation and site-directed mutagenesis studies were also carried out to investigate the binding mode of the most potent inhibitor **13h** in the active site of CBR1.

2. Results and Discussion

2.1. Inhibition of human CBR1 by chromene-2-carboxamide derivatives

CBR1 is potently inhibited by flavonoids with 7-hydroxylated chromene rings.[27] Since compound **1**, an inhibitor of AKR1B10 and AKR1B1,[31] has a 7-hydroxylated chromene ring, we first examined the inhibition of the isatin reductase activity of human CBR1 by **1** and its derivatives hydroxylated at different positions on the chromene ring (**2**-**4**), in our search for new inhibitors of this enzyme (Table 1). Compounds **1** and **2** inhibited CBR1, and their IC_{50} values are comparable to those of zearalenone analogue 5[23] and flavonoids other than luteolin.[27] Next, we tested the inhibitory activities of compounds **6** – **9**, which are synthetic intermediates of **1** – **4** and lack the 4-methoxyphenyl moiety. While **6** and **7** showed less inhibitory potency than the corresponding compounds with the 4-methoxyphenyl moiety (**1** and **2**, respectively), **9** with 8-hydroxychromene ring inhibited CBR1 with an IC_{50} value of 0.32 μM, and did not show significant inhibition for AKR1B10 and AKR1B1 at its high concentration of 10 μM. The results suggest that **9** binds to CBR1 differently from **1** and **2** due to the lack of the 4-methoxyphenyl moiety and its 8-hydroxyl group is a structural requisite for the selective inhibition of CBR1.

2.2. Synthesis of 8-hydroxychromene and 8-hydroxycoumarin derivatives

Using the selective CBR1 inhibitor **9** as the lead compound, we synthesized 8-hydroxy-2-iminochromene (**13a–13q**) and 8-hydroxycoumarin (**14a–14q**) derivatives by replacing the pyridine moiety bound to the carboxamide of the chromene ring with substituted phenyl or benzyl rings according to Scheme 1. The cyanoacetoamides (**12l**) and (**12m**) were prepared by the deprotection of the corresponding MOM-protected cyanoacetoamides, which were prepared by the condensation of benzylamines (**10l**) and (**10m**) with cyanoacetic acid by using EDC. The Knoevenagel condensation of cyanoacetoamides (**12a–12q**)

with 2,3-dihydroxybenzaldehyde (**11**) gave rise to the corresponding chromenes (**13a–13q**), [34] which were converted to the coumarins (**14a–14q**) by acid hydrolysis. To confirm the importance of the 8-hydroxy group on the chromene ring, compound **15** (i.e., 8-methoxy analog of **13h**) was also synthesized using 2-hydroxy-3-methoxybenzaldehyde instead of **11**.

2.3. Inhibitory potency and selectivity of the synthesized compounds

The structures of the synthesized compounds and their inhibitory activities for CBR1 are summarized in Table 2. In the 8-hydroxy-2-iminochromene derivatives, **13a** with a phenyl ring on the carboxamide showed slightly lower IC_{50} value than the lead **9**, but other derivatives having hydroxyphenyl and fluorophenyl rings (**13b**, **13d, 13e–13g**), except for **13c**, did not improve the inhibitory potency. In contrast, the derivatives having chlorophenyl rings (**13h–13j**) potently inhibited CBR1, and particularly the IC_{50} value (0.034 μM) of **13h** having 2-chlorophenyl ring was the lowest. Among the derivatives having benzyl and substituted benzyl rings, **13p** having 3-chlorobenzyl ring was the most potent inhibitor, although its IC_{50} value (0.090 μM) was higher than that of **13h**. For the 8-hydroxycoumarin derivatives (**14a–14q**), their IC_{50} values were higher than those of the 8-hydroxy-2-iminochromene derivatives having the corresponding substituents on the carboxamide moiety. For example, the most potent coumarin derivative was **14h**, but its IC_{50} value was 7-fold higher than that of the corresponding chromene derivative **13h**. Since the 8-hydroxy-2-iminochromene and 8-hydroxycoumarin derivatives differ only in the substituent at 2-position on the benzofuran ring (*i.e.*, imino or carbonyl group), the above results suggest that the 2-imino group is a structural requisite for the tight binding of **13h** and **13p**. It should be noted that an 8-methoxylated derivative of **13h** (**15**) did not inhibit CBR1 at its high concentration of 10 μM, being indicative of the most critical prerequisite of the 8-hydroxyl group for the CBR1 inhibition by these inhibitors.

Among the newly synthesized inhibitors, **13h** was the most potent, followed by **13o** and **13p**. The IC_{50} value of **13h** is 3-fold lower than that of luteolin that was previously reported as the most potent CBR1 inhibitor,[27] although those of **13o** and **13p** are comparable to that of luteolin. We examined the inhibitory selectivity of **13h**, **13o** and **13p** by comparing their effects on the activities of CBR3, DCXR, DHRS4 and AKRs (1B1, 1B10, 1C1, 1C2 and 1C4) that metabolized endogenous and xenobiotic carbonyl compounds[1,2,15] (Table 3). These inhibitors did not significantly inhibit the eight enzymes at a high concentration of 10 μM, with the exception of moderate inhibition of CBR3 (by **13h** and **13o)** and AKR1B10 (by **13o** and **13p)**. With respect to selectivity ratio (IC_{50} for the eight enzymes/that for CBR1), **13h** is the most selective inhibitor among the three synthesized compounds. To compare the inhibitory selectivity of **13h** with that of luteolin, we examined its inhibitory effects on the activities of the five enzymes. Luteolin inhibited all the eight enzymes, and its inhibitory selectivity was much lower than that of **13h**. Thus, **13h** is not only the most potent inhibitor among the known CBR1 inhibitors, but also highly selective.

2.4. Structural insight into high inhibitory potency and selectivity of 13h

The inhibition patterns of **13h** were examined in both NADPH-linked isatin reduction and $NADP^+$-linked (*S*)-(+)-1,2,3,4-tetrahydro-1-naphthol (*S*-tetralol) oxidation by CBR1. As reported for the inhibition patterns of luteolin,[27] the inhibition by **13h** was mixed-type with respect to isatin in the reduction reaction (K_i 64 ± 7 nM and K_i' 18 ± 2 nM), and was competitive with respect to *S*-tetralol in the oxidation reaction. The inhibition by **13p** was also competitive with respect to the substrate in the *S*-tetralol oxidation. The K_i values for **13h** and **13p** estimated in the *S*-tetralol oxidation were 15 and 47 nM, respectively (Table 4), of which the value for **13h** is 4-fold lower than that for luteolin (59 nM).[27] The inhibition patterns suggest that **13h** and **13p** bind to the substrate-binding site of CBR1.

The crystallographic and site-directed mutagenesis studies of human CBR1 and CBR3 have shown that despite their high sequence identity, the active sites of the two enzymes differ in shape and surface properties.[7] The residue difference in the substrate binding sites of the two enzymes is suggested to be related to their differences in substrate specificity. Such residues of CBR1 are Met141, Trp229 and Ala235 (Fig. 2), which correspond to Gln, Pro and Asp, respectively, in CBR3. The crystal structure of the CBR1-$NADP^+$-Hydroxy-PP complex has also shown that the pyrazolopyrimidine of the inhibitor is surrounded by hydrophobic residues (Ile140, Met141 and Trp229) and its phenolic hydroxyl group interacts with Ser139 and Tyr193 of the catalytic triad (supplementary Fig. S1).[22] To ensure the binding of **13h** to the substrate-binding site of CBR1, Met141 and Trp229 were chosen among the residues in the site, and subjected to site-directed mutagenesis (Table 4). Met141 was mutated to Gln (the corresponding residue of CBR3) and Val (a smaller hydrophobic residue), and Trp229 was mutated to Phe (a smaller aromatic residue) and Leu (a non-aromatic and hydrophobic residue). The Met141Gln mutation decreased the K_i (*i.e.*, affinity) for **13h** by 12-fold compared to wild-type CBR1, although the Met141Val mutation gave a smaller effect. The affinity for **13h** was impaired greatly by the Trp229Leu mutation, but the Trp229Phe mutation resulted in only 5-fold decrease. The results demonstrated that **13h** indeed binds to the substrate-binding site of CBR1, and suggest that the residue differences at positions 141 and 229 are related to the high inhibitory selectivity of **13h** to CBR1 over CBR3. The affinity for **13p** was similarly decreased by these mutations, but the effects of the mutagenesis of Trp229 were lower than those on the affinity for **13h**.

The underlying structural reasons for the high inhibitory potency and selectivity of **13h** were also examined by constructing models of docked **13h** in the CBR1-$NADP^+$ complex. In the model with the lowest IFDScore (Fig. 2), **13h** was held in the substrate-binding site through a number of contacts including the catalytic residues, Tyr193 and Ser139, of which the following interactions may be involved in the inhibitory potency and selectivity. 1) The 2-imino group on the chromene ring of **13h** formed a H-bond with Tyr193, which is probably related to the higher inhibitory potency of the 2-iminochromene derivatives than the coumarin derivatives. 2) A H-bond network was observed between **13h** (its 8-hydroxy group and 1-ether oxygen of the chromene ring) and CBR1 residues (the side chain of Ser139 and the main chain nitrogen of Ile140). In particular, the H-bond interaction of the 8-hydroxy group with the residues is probably important for the high inhibitory potency of **13h**, because its 8-methoxylated derivative (**15**) did not inhibit CBR1. 3) The chromene ring of **13h** was surrounded by hydrophobic side chains of Trp229, Met141 and Leu140, of which the side chain of Trp229 formed hydrophobic and π-stacking interactions. The indole nitrogen of Trp229 was in close proximity to the carbonyl group in the carboxamide of **13h** (2.9 Å), and can form an H-bond interaction. The importance of the interactions of Trp229 and Met141 with the chromene ring for the inhibitory potency of **13h** is supported by the larger impairment of its affinity by the Trp229Leu and Met141Gln mutations. 4) The 2-chlorophenyl moiety of **13h** was surrounded by the side and/or main chains of Ala93, Phe94, Lys95, Val96, Met141, Met234 and Ala235, and its chlorine atom was accommodated in a space composed of the side-chains Met234 and Ala235, although it was partially exposed to the solvent. Such interactions of this moiety might not be properly formed in the cases of other less potent derivatives with 3- and 4-chlorophenyl rings, and thereby affects the interactions of the other parts with the residues described above.

2.5. Inhibitory effects of 13h and 13p on cellular metabolism by CBR1

The efficacies of **13h** and **13p** as CBR1 inhibitors were investigated by analyzing their effects on cellular metabolism of 9,10-phenanthrenequinone (9,10-PQ) by human CBR1 overexpressed in bovine aorta endothelial cells (BAEC). 9,10-PQ is an efficient substrate of human CBR1,[9] and its reduction of 9,10-PQ generates its semiquinone, superoxide anion and hydrogen peroxide through the reaction of the reduced hydroquinone with molecular oxygen followed by redox cycling.[9,35,36] CBR4 and DCXR reduce 9,10-PQ and their overexpression in BAEC accelerates the generation of reactive oxygen species and cytotoxicity by 9,10-PQ.[4,37] However, there has been no report on the involvement of human CBR1 in the toxic effect of 9,10-PQ at cellular levels. Therefore, we first examined the effect of overexpression of CBR1 on cytotoxicity of 9,10-PQ using BAEC transfected with the cDNA. Compared to the control cells, the cytotoxicity of 9,10-PQ was clearly increased in the CBR1-transfected cells (Fig. 3A), indicating that the reduction of 9,10-PQ by the enzyme is indeed responsible for the cytotoxicity. Next, the inhibitory effects of **13h** and **13p** on the cellular metabolism of 9,10-PQ were assessed by determining the viability of the cells treated with 5 μM 9,10-PQ (Fig. 3B). While the two compounds had no effect on the viability of the control cells at 10 μM, they decreased the 9,10-PQ-induced cytotoxicity of the CBR1-overexpressing cells at their lower concentrations of 4 μM. Thus, the results indicate that, although **13h** and **13p** inhibited the cellular metabolism of 9,10-PQ by CBR1, **13p** is more effective than **13h** in the cells in contrast to their in vitro inhibitory potencies. This inconsistency between the results of in vitro and ex vivo experiments might have resulted from the differences in lipophilicities of **13h** and **13p**, although further studies on cell permeability are needed.

3. Experimental section

3.1. Chemistry

The chromenes (**13a–13q**), **15**, and the coumarins (**14a–14q**) were synthesized as shown in Scheme 1.

3.1.1. General

Melting points were determined with a Yanaco micro melting apparatus and were uncorrected. Flash chromatography was performed on Kanto Kagaku silica gel 60N. NMR spectra were recorded on JEOL GX 400 and JEOL JNX-ECX500 spectrometers. Chemical shifts (δ) are given in ppm downfield from TMS and referenced to $CHCl_3$ (7.26 ppm) or DMSO (2.50 ppm) for ^{1}H-NMR spectra and the centre line of $CDCl_3$ (77.0 ppm) for ^{13}C-NMR spectra as an internal standard. Peak multiplicities are designated by the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad and coupling constants in (*J*) Hz. High-resolution mass spectral data were obtained on a JEOL MStation JMS-700. All commercial reagents were used as received unless otherwise noted. The amides **12c**, **12i**, **12j**, **12k**, **12n**, **12o**, **12p**, and **12q** are commercially available from UORSY Building Blocks, Ukrine.

3.1.2. General procedure for the preparation of 12

To a stirred solution of cyanoacetic acid (1 mmol) in CH_2Cl_2 (5 mL) were added EDC (2 mmol), DMAP (0.2 mmol), and corresponding amine (1 mmol), and the resulting mixture was stirred for 24 h at room temperature. The volatiles were removed under reduced pressure, and the residue was chromatographed on silica gel (15 g, hexane : acetone = 5 : 1) to give the corresponding amide. The amides **12a**, **12d**, **12e**, **12f**, **12g**, **12h** are known compounds,[38] and the ^{1}H-NMR spectra of our synthetic materials were identical with those for the reported values.

***3.1.2.1. 2-Cyano-N-(2-methoxymethoxybenzyl)acetamide*.** Yield: 45%; mp: 86-88 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 3.33 (2H, s), 3.48 (3H, s), 4.47 (2H, d, *J* = 6.5 Hz), 5.24 (2H, s), 6.82 (1H, br), 6.97 (1H, td, *J* = 6.5, 1.0 Hz), 7.13 (1H, dd, *J* = 6.5, 1.0 Hz), 7.25 (1H, d, *J* = 6.5 Hz), 7.26 (1H, td, *J* = 6.5, 1.0Hz); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 25.9, 40.6, 56.5, 94.8, 114.3, 122.1, 125.7, 126.5, 129.7, 130.2, 155.6, 160.5; IR (KBr): 3291, 1653, 1559, 1155 cm^{-1}; MS (EI): m/z 234 (M^+); HRMS: Calcd for $C_{12}H_{14}N_2O_3$ 234.1004, Found: 234.1003.

***3.1.2.2. 2-Cyano-N-(3-methoxymethoxybenzyl)acetamide*.** Yield: 51%; mp: 83-85 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 3.41 (2H, s), 3.48 (3H, s), 4.45 (2H, d, *J* = 5.5 Hz), 5.17 (2H, s), 6.35 (1H, br), 6.93 (1H, d, *J* = 6.5 HZ), 6.96 (1H, s-like), 7.00 (1H, dd, *J* = 6.5, 2.5 Hz), 7.28 (1H, t, *J* = 6.5 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 25.5, 43.7, 55.8, 94.1, 114.8, 115.1, 115.4, 120.8, 129.6, 138.6, 157.2, 161.9; IR (KBr): 3288, 1646, 1555, 1155, 1030 cm^{-1}; MS (EI): m/z 234 (M^+); HRMS: Calcd for $C_{12}H_{14}N_2O_3$ 234.2512, Found: 234.0999.

Deprotection of MOM group

To a stirred solution of the acetoamides obtained above (1 mmol) in THF (5 mL) was added 10% HCl (5 drops), then the resulting mixture was heated to 40 °C for 24 h. After cooling, the reaction was quenched by H_2O (5 mL) and the aqueous mixture was extracted with EtOAc (5 mL x 3). The organic extracts were combined, dried over Na_2SO_4, and evaporated under reduced pressure. The residue was chromatographed on silica gel (15 g, hexane : acetone = 2 : 1) to give the corresponding amide.

3.1.2.3. 2-Cyano-N-(2-hydroxybenzyl)acetamide (12l). Yield: 91%; mp: 108-110 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 3.43 (2H, s), 4.43 (2H, d, *J* = 6.5 Hz), 6.88 (1H, td, *J* = 6.5, 1.0 Hz), 6.93 (1H, dd, *J* = 6.5, 1.0 Hz), 6.98 (1H, br), 7.14 (1H, dd, *J* = 6.5, 1.0 Hz), 7.23 (1H, td, *J* = 6.5, 1.0 Hz), 7.88 (1H, br); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 25.6, 40.8, 114.3, 117.3, 120.4, 123.0, 130.2, 130.8, 155.1, 163.2; IR (KBr): 3291, 1674, 1458 cm^{-1}; MS (EI): m/z 190 (M^+); HRMS: Calcd for $C_{10}H_{10}N_2O_3$ 190.0742, Found: 190.0741.

3.1.2.4. 2-Cyano-N-(3-hydroxybenzyl)acetamide (12m). Yield: 74%; mp: 85-87 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 3.41 (2H, s), 4.43 (2H, d, *J* = 6.0 Hz), 6.40 (1H, br), 6.77 (1H, s), 6.78 (1H, dd, *J* = 7.5, 1.0 Hz), 6.83 (1H, d, *J* = 7.5 Hz), 7.22 (1H, t, *J* = 7.5 Hz); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 25.8, 43.2, 114.5, 114.8, 116.8, 118.5, 129.9, 140.4, 158.0, 162.6; IR (KBr): 3209, 1653, 1558 cm^{-1}; MS (EI): m/z 190 (M^+); HRMS: Calcd for $C_{10}H_{10}N_2O_2$ 190.0742, Found 190.0741.

3.1.3. General procedure for the preparation of 13

To a stirred solution of cyanoamide **12** (1 mmol) in ethanol (5 mL) were added **11** (1 mmol), and piperidine (3 drops), and the resulting mixture was stirred for 24 h at room temperature. The solid product was filtrated, washed with ethanol, and dried to give corresponding chromene **13**.

3.1.3.1. 8-Hydroxy-2-imino-2H-chromene-3-carboxylic acid phenylamide (13a). Yield: 82%; mp: 235-237 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 7.07 (1H, d, *J* = 3.0 Hz), 7.08 (1H, d, *J* = 4.0 Hz), 7.11 (1H, t, *J* = 7.5 Hz), 7.23 (1H, dd, *J* = 4.0, 3.0 Hz), 7.37 (2H, t, *J* = 7.5 Hz), 7.67 (2H, dd, *J* = 7.5, 1.0 Hz), 8.50 (1H, d, *J* = 1.5 Hz), 9.09 (1H, s), 10.23 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 119.9, 120.1, 120.2, 120.4, 120.5, 124.5, 124.6, 129.6, 138.8, 142.5, 142.6, 144.5, 156.5, 160.3; IR (KBr): 3298, 1674, 1598, 1473 cm^{-1}; MS (EI): m/z 280 (M^+); HRMS: Calcd for $C_{16}H_{12}N_2O_3$ 280.0848, Found 280.0847.

3.1.3.2. 8-Hydroxy-2-imino-2H-chromene-3-carboxylic acid (2-hydroxyphenyl)amide (13b). Yield: 48%; mp: 253-255 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 6.80 (1H, td, *J* = 6.0, 2.0 Hz), 6.88-6.92 (1H, 1H, and 1H, each d), 7.06 (1H, d, *J* = 2.5 Hz), 7.07 (1H, d, *J* = 4.5 Hz), 7.21 (1H, dd, *J* = 4.5, 2.5 Hz), 8.37 (1H, d, *J* = 8.0 Hz), 8.48 (1H, s), 8.98 (1H, s), 9.96 (1H, br), 10.20 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 115.23, 119.5, 119.97, 120.05, 120.5, 121.0, 124.5, 124.6, 127.5, 142.0, 142.4, 142.5, 144.4, 147.7, 155.8, 160.1; IR (KBr): 3313, 1668, 1558, 1471 cm^{-1}; MS (EI): m/z 296 (M^+); HRMS: Calcd for $C_{16}H_{12}N_2O_4$ 296.0797, Found: 296.0798.

3.1.3.3. 8-Hydroxy-2-imino-2H-chromene-3-carboxylic acid (3-hydroxyphenyl)amide (13c). Yield: 40%; mp: 202-204 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 6.52 (1H, dd, *J* = 7.0, 2.0 Hz), 6.97 (1H, dd, *J* = 7.0, 1.0 Hz), 7.08 (1H, d, *J* = 2.5 Hz), 7.09 (1H, d, *J* = 4.5 Hz), 7.14 (1H, t, *J* = 7.0 Hz), 7.22 (1H, dd, *J* = 4.5, 2.5 Hz), 7.28 (1H, t, *J* = 2.0 Hz), 8.48 (1H, s), 9.08 (1H, s), 9.50 (1H, br), 10.20 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 107.1, 110.8, 111.7, 119.9, 120.2, 120.5, 120.6, 124.6, 130.3, 139.8, 142.5, 144.5, 156.5, 158.4, 160.1; IR (KBr): 3303, 1676, 1577, 1473 cm^{-1}; MS (EI): m/z 296 (M^+); HRMS: Calcd for $C_{16}H_{12}N_2O_3$ 296.0797, Found: 296.0799.

3.1.3.4. 8-Hydroxy-2-imino-2H-chromene-3-carboxylic acid (4-hydroxyphenyl)amide (13d). Yield: 46%; mp: 231-233 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 6.76 (2H, d, *J* = 7.8 Hz), 7.07 (1H, d, *J* = 3.0 Hz), 7.08 (1H, d, *J* = 3.5 Hz), 7.21 (1H, dd, *J* = 3.5, 3.0 Hz), 7.47 (2H, d, *J* = 7.8 Hz), 8.46 (1H, s), 9.03 (1H, s), 9.34 (1H, br), 12.58 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 116.0, 119.96, 120.03, 120.5,

120.7, 121.7, 124.6, 130.5, 142.1, 142.4, 144.5, 154.5, 156.5, 159.6; IR (KBr): 3287, 1669, 1513, 1473 cm^{-1}; MS (EI): m/z Calcd for $C_{16}H_{12}N_2O_4$ 296.0797, Found: 296.0796.

3.1.3.5. 8-Hydroxy-2-imino-2H-chromene-3-carboxylic acid (2-fluorophenyl)amide (13e). Yield: 82%; mp: 247-249 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 7.10 (1H, d, *J* = 8.0 Hz), 7.11 (1H, d, *J* = 10.0 Hz), 7.13-7.18 (1H, m), 7.23 (1H, d, *J* = 8.0 Hz), 7.26 (1H, dd, *J* = 8.0, 1.5 Hz), 7.33 (1H, dd, *J* = 10. 0, 8.0 Hz), 8.48 (1H, td, *J* = 8.0, 1.5 Hz), 8.55 (1H, d, *J* = 1.5 Hz), 9.14 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ115.6 (d, *J* = 23.1 Hz), 119.8, 120.2 (d, *J* = 23.1 Hz), 120.7, 121.9, 124.6, 125.0 (d, *J* = 8.5 Hz), 125.2 (d, *J* = 3.6 Hz), 127.0 (d, *J* = 8.5 Hz), 142.5, 143.1, 144.5, 153.0 (d, *J* = 241.8 Hz), 156.3, 160.6; IR (KBr): 3310, 1607, 1569, 1473 cm^{-1}; MS (EI): m/z 298 (M^+); HRMS: Calcd for $C_{16}H_{11}FN_2O_3$ 298.0754, Found 298.0754.

3.1.3.6. 8-Hydroxy-2-imino-2H-chromene-3-carboxylic acid (3-fluorophenyl)amide (13f). Yield: 51%; mp: 225-227 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 6.96 (1H, td, *J* = 7.0, 1.5 Hz), 7.06 (1H, d, *J* = 2.5 Hz), 7.08 (1H, d, *J* = 4.5 Hz), 7.22 (1H, dd, *J* = 4.5, 2.5 Hz), 7.28 (1H, d, *J* = 9.0 Hz), 7.39 (1H, q, *J* = 7.0 Hz), 7.74 (1H, d, *J* = 9.0 Hz), 8.50 (1H, s), 9.10 (1H, br), 10.22 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 107.0 (d, *J* = 23.1 Hz), 110.0 (d, *J* = 23.1 Hz), 116.0, 119.8, 120.1, 120.3, 120.6, 124.6 (d, *J* = 1.3 Hz), 131.1 (d, *J* = 10.3 Hz), 140.4 (d, *J* = 10.3 Hz), 142.5, 142.9, 144.5, 156.4, 160.7, 162.8 (d, *J* = 239.4 Hz); IR (KBr): 3305, 1609, 1579, 1473 cm^{-1}; MS (EI): m/z 298 (M^+); HRMS: Calcd for $C_{16}H_{11}FN_2O_3$ 298.0754, Found 298.0754.

3.1.3.7. 8-Hydroxy-2-imino-2H-chromene-3-carboxylic acid (4-fluorophenyl)amide (13g). Yield: 65%; mp: 240-242 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 7.09-7.10 (1H and 1H, each d), 7.22-7.24 (2H and 1H, each dd), 7.71 (1H, dd, *J* = 5.0, 4.5 Hz), 8.50 (1H, s), 9.09 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 116.1 (d, *J* = 23.1 Hz), 119.8, 120.2, 120.3, 120.5, 121.9 (d, *J* = 7.4 Hz), 124.6 (d, *J* = 3.6 Hz), 135.2, 142.4, 142.6, 144.5, 156.4, 158.9 (d, *J* = 239.0 Hz), 160.3; IR (KBr): 3298, 1674, 1511,1218 cm^{-1}; MS (EI): m/z 298 (M^+); HRMS: Calcd for $C_{16}H_{11}FN_2O_3$ 298.0754, Found 298.0754.

3.1.3.8. 8-Hydroxy-2-imino-2H-chromene-3-carboxylic acid (2-chlorophenyl)amide (13h).

Yield: 67%; mp: 282-283 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ; 7.08-7.13 (1H, 1H, and 1H, each d), 7.16 (1H, td, *J* = 6.5, 1.5 Hz), 7.24 (1H, dd, *J* = 5.0, 2.0 Hz), 7.39 (1H, td, *J* = 7.0, 1.0 Hz), 7.54 (1H, dd, *J* = 6.5, 1.5 Hz), 8.52 (1H, dd, *J* = 7.0, 1.0 Hz), 8.55 (1H, s), 9.13 (1H, s); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 119.8, 120.2, 120.4, 120.7, 122.6, 123.6, 124.6, 125.5, 128.2, 129.9, 136.0, 142.5, 143.3, 144.5, 156.0, 160.8; IR (KBr): 3305, 1684, 1471, 1224 cm^{-1}; MS (EI): m/z 314 (M^+); HRMS: Calcd for $C_{16}H_{11}ClN_2O_3$ 314.0458, Found: 314.0459.

3.1.3.9. 8-Hydroxy-2-imino-2H-chromene-3-carboxylic acid (3-chlorophenyl)amide (13i). Yield: 65%; mp: 252-254 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 7.07-7.13 (1H and 1H, each d), 7.18 (1H, d, *J* = 8.0 Hz), 7.23 (1H, d, *J* = 8.0 Hz), 7.39 (1H, t, *J* = 8.0 Hz), 7.44 (1H, d, *J* = 8.0 Hz), 7.95 (1H, s), 8.50 (1H, s), 9.12 (1H, s); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 118.6, 119.6, 119.8, 120.1, 120.3, 120.6, 124.2, 124.6, 131.2, 133.9, 140.1, 142.5, 142.9, 144.5, 156.4, 160.7; IR (KBr): 3301, 1681, 1595, 1482 cm^{-1}; MS (EI): m/z 314 (M^+); HRMS: Calcd for $C_{16}H_{11}ClN_2O_3$ 314.0458, Found: 314.0458.

3.1.3.10. 8-Hydroxy-2-imino-2H-chromene-3-carboxylic acid (4-chlorophenyl)amide (13j). Yield: 91%; mp: 272-273 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 7.07-7.11 (1H and 1H, each d), 7.23 (1H, dd, *J* = 5.0, 2.5 Hz), 7.43 (2H, dd, *J* = 5.0, 2.0 Hz), 7.70 (2H, dd, *J* = 5.0, 2.0 Hz), 8.50 (1H, d, *J* = 1.5 Hz), 9.11 (1H, s); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 119.8, 120.2, 120.3, 120.6, 121.7, 124.6, 128.1, 129.5, 137.7, 142.5, 142.7, 144.5, 156.4, 160.5; IR (KBr): 3302, 1675, 1473, 1230 cm^{-1}; MS (EI): m/z 314 (M^+); HRMS: Calcd for $C_{16}H_{11}ClN_2O_3$ 314.0458, Found: 314.0459.

3.1.3.11. 8-Hydroxy-2-imino-2H-chromene-3-carboxylic acid benzylamide (13k). Yield: 55%; mp: 206-208 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 4.53 (2H, d, *J* = 6.0 Hz), 7.04-7.08 (1H and 1H,each d), 7.18 (1H, dd, *J* = 8.0, 6.0 Hz), 7.25-7.28 (1H, m), 7.32-7.36 (4H, m), 8.39 (1H, s), 8.82 (1H, s), 10.74 (1H, t, *J* = 6.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 43.3, 119.9, 120.4, 120.5, 124.4, 127.5, 127.9, 128.8, 129.0, 139.3, 141.9, 142.4, 144.4, 156.1, 162.2; IR (KBr): 3320, 1696, 1558, 1469 cm^{-1}; MS (EI): m/z 294 (M^+); HRMS: Calcd for $C_{17}H_{14}N_2O_3$ 294.1004, Found: 294.1000.

3.1.3.12. 8-Hydroxy-2-imino-2H-chromene-3-carboxylic acid 2-hydroxybenzylamide (13l). Yield: 62%; mp: 140-141 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 4.30 (2H, d, *J* = 6.0 Hz), 6.76 (1H, t, *J* = 7.0 Hz), 6.82 (1H, d, *J* = 7.0 Hz), 7.03-7.06 (1H and 1H, each d), 7.09 (1H, dd, *J* = 5.0, 2.5 Hz), 7.17 (2H, dd, *J* = 7.0, 4.0 Hz), 8.38 (1H, s), 8.79 (1H, s), 10.66 (1H, t, *J* = 6.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 39.4, 115.4, 115.6, 119.4, 124.4, 125.7, 128.89, 128.94, 129.6, 141.9, 144.4, 145.0, 148.7, 155.8, 161.1, 161.7, 162.1; IR (KBr): 3309, 1669, 1457, 1240 cm^{-1}; MS (EI): m/z 310 (M^+); HRMS: Calcd for $C_{17}H_{14}N_2O_4$ 310.0954, Found: 310.0951.

3.1.3.13. 8-Hydroxy-2-imino-2H-chromene-3-carboxylic acid 3-hydroxybenzylamide (13m). Yield: 46%; mp: 205-207 °C; ^{1}H-NMR

(500 MHz, DMSO-d_6) δ 4.44 (2H, d, *J* = 6.0 Hz), 6.64 (1H, dd, *J* = 6.0, 2.0 Hz), 6.69-6.74 (1H and 1H, d and s), 7.04-7.08 (1H and 1H, each d), 7.12 (1H, t, *J* = 8.0 Hz), 7.19 (1H, dd, *J* = 7.0, 4.0 Hz), 8.39 (1H, s), 8.82 (1H, br), 10.70 (1H, t, *J* = 5.5 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 43.2, 114.5, 114.7, 118.4, 119.8, 119.9, 120.4, 124.4, 130.0, 140.7, 141.9, 142.4, 144.4, 156.1, 158.0, 162.1; IR (KBr): 3291, 1669, 1596, 1473 cm^{-1}; MS (EI): m/z 310 (M^+); HRMS: Calcd for $C_{17}H_{14}N_2O_4$ 310.0954, Found: 310.0954.

3.1.3.14. 8-Hydroxy-2-imino-2H-chromene-3-carboxylic acid 4-hydroxybenzylamide (13n). Yield: 24%; mp: 187-188 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 4.38 (2H, d, *J* = 6.0 Hz), 6.72 (2H, d, *J* = 8.0 Hz), 7.05-7.07 (1H and 1H, each d), 7.12 (2H, d, *J* = 8.0 Hz), 7.18 (1H, dd, *J* = 3.5, 3.0 Hz), 8.39 (1H, s), 8.78 (1H, br), 10.61 (1H, t, *J* = 6.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 42.9, 115.5, 115.8, 119.9, 120.4, 120.6, 124.4, 129.4, 141.9, 142.4, 144.4, 156.1, 157.0, 160.6, 161.9; IR (KBr): 3293, 1675, 1473, 1240 cm^{-1}; MS (EI): m/z 310 (M^+); HRMS: Calcd for $C_{17}H_{14}N_2O_3$ 310.0954, Found: 310.0951.

3.1.3.15. 8-Hydroxy-2-imino-2H-chromene-3-carboxylic acid 2-chlorobenzylamide (13o). Yield: 85%; mp: 258-259 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 4.59 (2H, d, *J* = 6.0 Hz), 7.04-7.08 (1H and 1H, each d), 7.18 (1H, dd, *J* = 4.0, 3.0 Hz), 7.32-7.35 (2H, m), 7.41-7.43 (1H, m), 7.47-7.49 (1H, m), 8.38 (1H, s), 8.84 (1H, s), 10.81 (1H, t, *J* = 6.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 41.4, 119.8, 120.0, 120.37, 120.43, 124.5, 127.9, 129.5, 129.8, 129.9, 132.9, 136.4, 142.1, 142.5, 144.4, 156.1, 162.3; IR (KBr): 3301, 1674, 1472, 1225 cm^{-1}; MS (EI): m/z 328 (M^+); HRMS: Calcd for $C_{17}H_{13}ClN_2O_3$ 328.0615, Found: 328.0618.

3.1.3.16. 8-Hydroxy-2-imino-2H-chromene-3-carboxylic acid 3-chlorobenzylamide (13p). Yield: 90%; mp: 229-230°C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 4.53 (2H, d, *J* = 5.5 Hz), 7.04-7.08 (1H and 1H, each d), 7.18 (1H, dd, *J* = 4.0, 3.0 Hz), 7.30 (1H, d-like, *J* = 7.0 Hz), 7.33 (1H, d-like, *J* = 7.0 Hz), 7.36-7.40 (2H, m), 8.39 (1H, s), 8.85 (1H, br), 10.77 (1H, t, *J* = 6.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 42.7, 119.8, 120.0, 120.4, 124.4, 126.6, 127.4, 127.5, 127.7, 130.8, 133.6, 142.1, 142.2, 142.5, 144.5, 156.1, 162.4; IR (KBr): 3299, 1674, 1472, 1225 cm^{-1}; MS (EI): m/z 328 (M^+); HRMS: Calcd for $C_{17}H_{13}ClN_2O_3$ 328.0615, Found: 328.0612.

3.1.3.17. 8-Hydroxy-2-imino-2H-chromene-3-carboxylic acid 4-chlorobenzylamide (13q). Yield: 93%; mp: 241-242 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 4.53 (2H, d, *J* = 6.0 Hz), 7.03-7.08 (1H and 1H, each d), 7.18 (1H, dd, *J* = 4.0, 2.5 Hz), 7.35 (2H, d, *J* = 7.0 Hz), 7.40 (2H, d, *J* = 7.0 Hz), 8.39 (1H, s), 8.94 (1H, s), 10.75 (1H, t, *J* = 6.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 42.6, 119.8, 120.0, 120.4, 120.5, 124.4, 128.9, 129.8, 132.1, 138.6, 142.0, 142.5, 144.5, 156.2, 162.3; IR (KBr): 3299, 1675, 1473, 1229 cm^{-1}; MS (EI): m/z 328 (M^+); HRMS: Calcd for $C_{17}H_{13}ClN_2O_3$ 328.0615, Found: 328.0612.

3.1.3.18. 8-Methoxy-2-imino-2H-chromene-3-carboxylic acid (2-chlorophenyl)amide (15). This compound was prepared by the same procedure for **13** using 2-hydroxy-3-methoxybenzaldehyde instead of **11**.

Yield: 98%; mp: 237-238 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 3.89 (3H, s), 7.16 (1H, t, *J* = 7.0 Hz), 7.22 (1H, t, *J* = 7.0 Hz), 7.31 (1H, d, *J* = 7.0 Hz), 7.36-7.40 (3H, m), 7.53 (1H, d, *J* =7.3 Hz), 8.49 (1H, d, *J* = 7.3 Hz), 8.56 (1H, s), 9.36 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 56.3, 115.0, 119.0, 120.9, 121.2, 122.6, 124.0, 124.4, 124.8, 127.3, 129.3, 135.8, 142.6, 143.3, 146.3, 156.8, 160.5; IR (KBr): cm^{-1} MS (EI): m/z 328 (M^+); HRMS: Calcd for $C_{17}H_{13}ClN_2O_3$ 328.0615, found: 328.0618.

3.1.4. General procedure for the preparation of 14

To a stirred solution of chromene **13** (1 mmol) in THF (5 mL) was added 10% HCl (5 drops), and the resulting mixture was heated to 40 °C for 24 h. The reaction was quenched by H_2O (5 mL), and the aqueous mixture was extracted with EtOAc (5 mL x 3), the organic extracts were combined, and dried over Na_2SO_4, and the solvent was removed under reduced pressure. The residue was chromatographed on silica gel (15 g, CH_2Cl_2) to give the corresponding **14**.

3.1.4.1. 8-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid phenylamide (14a). Yield: 81%; mp: 251-253 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 7.15 (1H, td, *J* = 7.5, 1.5 Hz), 7.24-7.28 (1H and 1H, each d), 7.37-7.42 (3H, m), 7.72 (2H, dd, *J* = 7.5, 1.5 Hz), 8.86 (1H, s), 10.50 (1H, br), 10.69 (1H, s); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 120.0, 120.1, 120.4, 120.6, 120.8, 124.8, 125.8, 129.5, 138.5, 143.1, 145.0, 148.4, 160.4, 161.0; IR (KBr): 3424, 1707, 1598, 1472 cm^{-1}; MS (EI): m/z 281 (M^+); HRMS: Calcd for $C_{16}H_{11}NO_4$ 281.0688, Found 281.0689.

3.1.4.2. 8-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid (2-hydroxyphenyl)amide (14b). Yield: 81%; mp: 224-226 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 6.83 (1H, td, *J* = 7.0, 1.5 Hz), 6.92 (1H, dd, *J* = 7.0, 1.5 Hz), 6.97 (1H, td, *J* = 7.0, 1.5 Hz), 7.25-7.28 (1H and 1H, each d), 7.45 (1H, dd, *J* = 4.0, 3.0 Hz), 8.39 (1H, dd, *J* = 7.0, 1.5 Hz), 8.99 (1H, s), 10.02 (1H, br), 10.51 (1H, br), 11.14 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 115.1, 119.0, 119.7, 120.1, 120.4, 120.7, 120.9, 124.8, 125.7, 127.1, 143.2, 145.1, 147.2, 149.3, 159.4, 161.4; IR (KBr): 3177, 1718, 1558, 1457 cm^{-1}; MS (EI): m/z 297 (M^+); HRMS: Calcd for $C_{16}H_{11}NO_5$ 297.0637, Found 297.0634.

3.1.4.3. 8-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid (3-hydroxyphenyl)amide (14c). Yield: 83%; mp: 248-250 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 6.55 (1H, dd, J = 6.5, 1.5 Hz), 7.02 (1H, dd, *J* = 6.5, 1.5 Hz), 7.16 (1H, t, *J* = 6.5 Hz), 7.24-7.28 (1H and 1H, each d), 7.32 (1H, t, *J* = 3.0 Hz), 7.41 (1H, dd, *J* = 4.5, 2.5 Hz), 8.84 (1H, s), 9.54 (1H, s), 10.49 (1H, br), 10.61 (1H, s); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 107.3, 111.0, 111.9, 120.0, 120.2, 120.6, 120.8, 125.8, 130.2, 139.5, 143.0, 145.0, 148.3, 158.3, 160.2, 161.0; IR (KBr): 3288, 1714, 1609, 1472 cm^{-1}; MS (EI): m/z 297 (M^+); HRMS: Calcd for $C_{16}H_{11}NO_5$ 297.0637, Found 297.0639.

3.1.4.4. 8-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid (4-hydroxyphenyl)amide (14d).

Yield: 52%; mp: 254-256 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 6.75 (2H, d, *J* = 6.5 Hz), 7.23-7.25 (1H and 1H, each d, m), 7.39 (1H, dd, *J* = 5.0, 2.5 Hz), 7.50 (2H, d, *J* = 6.5 Hz), 8.83 (1H, s), 9.36 (1H, s), 10.47 (1H, s), 10.48 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 115.9, 120.00, 120.03, 120.6, 120.7, 122.1, 125.7, 130.1, 143.0, 145.0, 148.2, 154.7, 159.7, 161.1; IR (KBr): 3113, 1705, 1513, 1473 cm^{-1}; MS (EI): m/z 297 (M^+); HRMS: Calcd for $C_{16}H_{11}NO_5$ 297.0637, Found 297.0638.

3.1.4.5. 8-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid (2-fluorophenyl)amide (14e). Yield: 98%; mp: 248-250 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 7.20 (1H, m), 7.25-7.29 (3H m), 7.36 (1H, dd, *J* = 5.0, 2.5 Hz), 7.48 (1H, d, *J* = 6.5 Hz), 8.40 (1H, t, *J* = 6.5 Hz), 9.00 (1H, s), 10.53 (1H, br), 11.08 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 115.7 (d, *J* = 18.3 Hz), 118.7, 120.0, 120.9, 121.2, 122.1, 125.4 (d, *J* = 2.4 Hz), 125.5 (d, *J* = 8.4 Hz), 125.9, 126.5 (d, *J* = 8.6 Hz), 143.1, 145.1, 149.6, 152.9 (d, *J* = 241.9 Hz), 160.2, 161.7; IR (KBr): 3386, 1704, 1554, 1471 cm^{-1}; MS (EI): m/z 299 (M^+); HRMS: Calcd for $C_{16}H_{10}FNO_4$ 299.0594, Found 299.0591.

3.1.4.6. 8-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid (3-fluorophenyl)-amide (14f). Yield: 85%; mp: 247-249 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 6.99 (1H, tt, *J* = 6.5, 1.5 Hz), 7.26-7.29 (1H and 1H, each d), 7.42-7.46 (3H, m), 7.78 (1H, dt, *J* = 6.5, 1.5 Hz), 8.86 (1H, s), 10.52 (1H, br), 10.83 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 107.3 (d, *J* = 23.8 Hz), 111.3 (d, *J* = 23.8 Hz), 116.3, 119.9, 120.0, 120.6, 120.9, 125.8 (d, *J* = 1.25 Hz), 131.1 (d, *J* = 10.3 Hz), 140.1 (d, *J* = 10.3 Hz), 143.1, 145.0, 148.6, 160.7, 160.8, 162.7 (d, *J* = 239.3 Hz); IR (KBr): 3436, 1701, 1555, 1471 cm^{-1}; MS (EI): m/z 299 (M^+); HRMS: Calcd for $C_{16}H_{10}FNO_4$ 299.0594, Found 299.0591.

3.1.4.7. 8-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid (4-fluorophenyl)amide (14g). Yield: 89%; mp: 241-243 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 7.21-7.27 (4H, m), 7.41 (1H, dd, *J* = 4.5, 2.5 Hz), 7.56 (2H, dd, *J* = 6.5, 4.5 Hz), 8.85 (1H, s), 10.70 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 116.1 (d, *J* = 23.1 Hz), 119.9, 120.1, 120.6, 120.9, 122.3 (d, *J* = 8.5 Hz), 125.8 (d, *J* = 3.6 Hz), 134.9, 143.1, 145.1, 148.4, 159.1 (d, *J* = 239.4 Hz), 160.5, 160.8; IR (KBr): 3372, 1611, 1508, 1472 cm^{-1}; MS (EI): m/z 299 (M^+); HRMS: Calcd for $C_{16}H_{10}FNO_4$ 299.0594, Found 299.0591.

3.1.4.8. 8-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid (2-chlorophenyl)amide (14h). Yield: 95%; mp: 286-287 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 7.20 (1H, td, *J* = 7.0, 1.5 Hz), 7.28-7.29 (1H and 1H, each d), 7.42 (1H, td, *J* = 7.0, 1.5 Hz), 7.48 (1H, t, *J* = 5.5 Hz), 7.59 (1H, dd, *J* = 7.0, 1.5 Hz), 8.53 (1H, dd, *J* = 7.0, 1.0 Hz), 9.03 (1H, s), 10.56 (1H, br), 11.31 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 118.5, 120.0, 121.0, 121.3, 122.3, 123.3, 125.9, 128.5, 130.0, 135.3, 137.7, 143.2, 145.1, 150.0, 160.3, 161.7; IR (KBr): 3200, 1733, 1473, 1202 cm^{-1}; MS (EI): m/z 315 (M^+); HRMS: Calcd for $C_{16}H_{10}ClNO_4$ 315.0298, Found: 315.0301.

3.1.4.9. 8-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid (3-chlorophenyl)amide (14i). Yield: 47%; mp: 266-268 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 7.21 (1H, ddd, *J* = 7.0, 2.5, 1.0 Hz), 7.24-7.28 (1H and 1H, each d), 7.38-7.42 (2H, m), 7.57 (1H, ddd, J = 7.0, 2.5, 1.0 Hz), 7.90 (1H, d, *J* = 2.0 Hz), 8.83 (1H, s), 10.78 (1H, s); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 119.0, 119.8, 119.9, 120.0, 120.6, 120.9, 124.5, 125.8, 131.2, 133.8, 139.9, 143.1, 145.0, 148.6, 160.6, 160.9; IR (KBr): 3238, 1733, 1604, 1473 cm^{-1}; MS (EI): m/z 315 (M^+); HRMS: Calcd for $C_{16}H_{10}ClNO_4$ 315.0298, Found: 315.0301.

3.1.4.10. 8-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid (4-chlorophenyl)amide (14j). Yield: 54%; mp: 299-300 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 7.24-7.28 (1H and 1H, each d), 7.41 (1H, dd, *J* = 5.0, 2.5 Hz), 7.44 (2H, d, *J* = 7.0 Hz), 7.77 (2H, d, *J* = 7.0 Hz), 8.84 (1H, s), 10.76 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 119.9, 120.0, 120.5, 120.9, 122.0, 125.8, 128.4, 129.4, 137.4, 143.1, 145.2, 148.5, 160.7, 160.8; IR (KBr): 3233, 1733, 1472, 1207 cm^{-1}; MS (EI): m/z 315 (M^+); HRMS: Calcd for $C_{16}H_{10}ClNO_4$ 315.0298, Found: 315.0301.

3.1.4.11. 8-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid benzylamide (14k). Yield: 99%; mp: 262-264 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 4.54 (2H, d, *J* = 6.0 Hz), 7.22-7.40 (8H, m), 8.82 (1H, s), 9.14 (1H, t, *J* = 6.0 Hz), 10.45 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 119.3, 119.9, 120.56, 120.62, 125.6, 127.4, 127.9, 128.9, 139.4, 143.1, 144.9, 148.5, 160.8, 161.8; IR (KBr): 3320, 1672, 1583, 1473 cm^{-1}; MS (EI): m/z 295 (M^+); HRMS: Calcd for $C_{17}H_{13}NO_4$ 295.0845, Found: 295.0845.

3.1.4.12. 8-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid 2-hydroxybenzylamide (14l). Yield: 50%; mp: 256-257 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 4.47 (2H, d, *J* = 6.0 Hz), 6.76 (1H, td, *J* = 7.0, 0.5 Hz), 6.83 (1H, dd, *J* = 7.0, 0.5 Hz), 7.10 (1H, td, *J* = 7.0, 1.5 Hz), 7.19 (1H, dd, *J* = 7.0, 1.5 Hz), 7.22-7.24 (1H and 1H, each d), 7.39 (1H, dd, *J* = 4.0, 2.5 Hz), 8.84 (1H, s), 9.16 (1H, t, *J* = 6.0 Hz), 9.70 (1H, br), 10.45 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 39.4, 115.6, 119.0, 119.4, 120.0, 120.6, 120.7, 124.9, 125.6, 128.9, 129.6, 143.1, 145.0, 148.7, 155.8, 161.0, 161.6; IR (KBr): 3315, 1685, 1555, 1293 cm^{-1}; MS (EI): m/z 311 (M^+); HRMS: Calcd for $C_{17}H_{13}NO_5$ 311.0794, Found: 311.0793.

3.1.4.13. 8-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid 3-hydroxybenzylamide (14m). Yield: 81%; mp: 208-210 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 4.45 (2H, d, *J* = 6.0 Hz), 6.64 (1H, dd, *J* = 6.5, 2.5 Hz), 6.75 (1H, s), 6.77 (1H, d, *J* = 6.5 Hz), 7.12 (1H, t, *J* = 6.5 Hz), 7.21-7.26 (1H and 1H, each d), 7.39 (1H, dd, *J* = 5.0, 2.5 Hz), 8.82 (1H, s), 9.08 (1H, t, *J* = 6.0 Hz), 9.39 (1H, s), 10.47 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 114.4, 114.7, 118.5, 119.3, 119.9, 120.6, 120.7, 125.6, 129.9, 140.7, 143.1, 145.0, 148.6, 157.9, 160.8, 161.7; IR (KBr): 3320, 1700, 1539, 1473 cm^{-1}; MS (EI): m/z 311 (M^+); HRMS: Calcd for $C_{17}H_{13}NO_5$ 311.0794, Found: 311.0793.

3.1.4.14. 8-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid 4-hydroxybenzylamide (14n). Yield: 99%; mp: 234-235 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 4.41 (2H, d, *J* = 6.0 Hz), 6.72 (2H, d, *J* = 8.0 Hz), 7.16 (2H, d, *J* = 8.0 Hz), 7.20-7.25 (1H and 1H, each d), 7.38 (1H, dd, *J* = 5.0, 2.0 Hz), 8.82 (1H, s), 8.99 (1H, t, *J* = 6.0 Hz), 9.33 (1H, br), 10.44 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 31.2, 42.9, 115.7, 119.3, 120.0, 120.60, 120.65, 125.65, 129.5, 143.1, 145.0, 148.5, 157.0, 160.9, 161.6; IR (KBr): 3321, 1699, 1472, 1214 cm^{-1}; MS (EI): m/z 311 (M^+); HRMS: Calcd for $C_{17}H_{13}NO_5$ 311.0794, Found: 311.0795.

3.1.4.15. 8-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid 2-chlorobenzylamide (14o). Yield: 71%; mp: 248-249 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 4.61 (2H, d, *J* = 6.0 Hz) 7.22-7.26 (1H and 1H, each d), 7.31-7.34 (2H, m), 7.39 (1H, dd, *J* = 4.0, 2.5 Hz), 7.42 (1H, dd, *J* = 7.0, 2.5 Hz), 7.47 (1H, d-like, *J* = 7.0 Hz), 8.82 (1H, s), 9.21 (1H, t, *J* = 6.0 Hz), 10.45 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 119.1, 119.9, 120.6, 120.7, 125.7, 127.8, 129.3, 129.5, 129.7, 132.6, 136.4, 143.1, 145.0, 148.7, 160.9, 162.1; IR (KBr): 3329, 1701, 1470, 1248 cm^{-1}; MS (EI): m/z 329 (M^+); HRMS: Calcd for $C_{17}H_{12}ClNO_4$ 329.0455, Found: 329.0457.

3.1.4.16. 8-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid 3-chlorobenzylamide (14p). Yield: 90%; mp: 207-208 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 4.54 (2H, d, *J* = 6.0 Hz), 7.21-7.25 (1H and 1H, each d), 7.31-7.34 (2H, m), 7.37-7.39 (2H, m), 7.42 (1H, t-like, *J* = 2.0 Hz), 8.81 (1H, s), 9.21 (1H, t, *J* = 6.0 Hz), 10.44 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 42.7, 119.3, 119.9, 120.6, 120.7, 125.6, 126.6, 127.4, 127.8, 130.7, 133.5, 142.3, 143.1, 145.0, 148.6, 160.7, 162.1; IR (KBr): 3334, 1696, 1472, 1211 cm^{-1}; MS (EI): m/z 329 (M^+); HRMS: Calcd for $C_{17}H_{12}ClNO_4$ 329.0455, Found: 329.0454.

3.1.4.17. 8-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid 4-chlorobenzylamide (14q). Yield: 73%; mp: 269-270 °C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ 4.52 (2H, d, *J* = 6.0 Hz), 7.20-7.25 (1H and 1H, each d), 7.36-7.41 (5H, m, including at 7.39 (4H, d, J = 7.0 Hz)), 8.81 (1H, s), 9.18 (1H, t, *J* = 6.0 Hz), 10.45 (1H, br); ^{13}C-NMR (125 MHz, DMSO-d_6) δ 42.6, 119.3, 119.9, 120.6, 120.7, 125.6, 128.8, 129.8, 132.0, 138.6, 143.1, 145.0, 148.6, 160.7, 162.0; IR (KBr): 3320, 1694, 1468, 1204 cm^{-1}; MS (EI): m/z 329 (M^+); HRMS: Calcd for $C_{17}H_{12}ClNO_4$ 329.0455, Found: 329.0457.

3.2. Biological assays

3.2.1. Preparation of recombinant enzymes

The recombinant CBR1,[6] CBR3,[6] DHRS4,[30] DCXR,[33] AKR1B1,[16] AKR1B10,[39] AKR1C1,[40] AKR1C4[40] and AKR1C2[41] were prepared and purified to homogeneity, as described previously.

The recombinant CBR1,[6] CBR3,[6] DHRS4,[30] DCXR,[33] AKR1B1[16] and AKR1B10[38] were prepared and purified to homogeneity, as described previously. Site-directed mutagenesis was performed using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the pET28a expression plasmid harboring the cDNA for CBR1[6] as the template, according to the protocol described by the manufacturer. The primer pair used for the mutagenesis was composed of sense and antisense oligonucleotides to alter one codon of the cDNA. The 26- to 30-mer primers were synthesized to give the Met141Qln, Met141Val, Trp229Phe and Trp229Leu mutant enzymes (Supplementary Table S1). The coding regions of the cDNAs in the expression plasmids were sequenced by using a Beckman CEQ8000XL DNA sequencer in order to confirm the presence of the desired mutation and ensure that no other mutation had occurred. The expression constructs were transformed into *Escherichia coli* BL21 (DE3) pLysS cells (Life Technologies, Carlsbad, CA). The mutant enzymes were expressed in *Escherichia coli* cells, and purified to homogeneity as described above for the wild-type CBR1.[6]

3.2.2. Assay of enzyme activity

The reductase and dehydrogenase activities of CBR1 were assayed at 25 ˚C by measuring the rate of change in NADPH absorbance (at 340 nm) and fluorescence (at 455 nm with an excitation wavelength of 340 nm), respectively.[27] The IC_{50} values for inhibitors were determined in the reaction mixture that consisted of 0.1 M potassium phosphate buffer, pH 7.0, 0.1 mM NADPH, substrate and enzyme, in a total volume of 2.0 mL. The substrates were 50 μM isatin (for CBR1), 0.1 mM menadione (for CBR3), 0.4 mM diacetyl (for DCXR), 25 μM 1-phenylisatin (for DHRS4) and 0.2 mM pyridine-3-aldehyde (for AKR1B10 and AKR1B1). The dehydrogenase activity of CBR1 was assayed using 0.25 mM $NADP^+$ and an appropriate amount of *S*-tetralol as the coenzyme and substrate, respectively, in the above reaction mixture. The kinetic studies in the presence of inhibitors were carried out in both isatin reduction and *S*-tetralol oxidation over a range of five or six substrate concentrations at the above saturating concentration of NADPH or $NADP^+$.[27] The inhibition patterns were judged from the Lineweaver-Burk plots, and inhibition constants, K_i (slope effect) and K_i' (intercept effect), were determined from replots of the slopes and intercepts, respectively, versus inhibitor concentration.[41] The IC_{50} and K_i values are expressed as the means ± SD of at least three determinations.

3.2.3. Molecular modeling

All calculations were performed using the Schrödinger suite 2013-2 (Schrödinger, LLC, New York, NY). The 2D structure of **13h** was first converted into 3D structure using the LigPrep2.7 program. The protonation states of the compounds were predicted using Epik2.5 program.[43] Then, the conformational search of the compound was carried out using the ConfGen2.5 program,[44] and the resulting conformers were used in the following docking calculations.

In the docking calculations, a total of five X-ray structures of CBR1 with the following PDB codes were used in order to consider protein flexibility as much as possible: 1WMA,[7] 2PFG,[45] 3BHI, 3BHJ, and 3BHM.[12] The structures were prepared using the Protein Preparation Wizard in Maestro9.5. The missing atoms for side chains were compensated for using Prime3.3 program.[46] Finally, the structures were minimized using force-field OPLS 2005. We removed all HETATM molecules except for $NADP^+$ (i.e.: ligands, ions, and water molecules) before docking calculations. The docking calculation of **13h** against five X-ray structures of CBR1 were performed using the IFD 2006 protocol as implemented in Schrödinger suite 2013-2.[47] Box center for the "Receptor Grid Generation" protocol was set to a centroid of Ser139, Met141, Trp229, and Ala235 of CBR1, which were residues forming the active site. Initial Glide docking was performed using reduced van der Waals radii for protein and ligand to allow minor readjustments. The van der Waals radii of both molecules were scaled by 80%. The maximum number of poses per conformer was set to 2. Prime was used to refine residues within 5.0 Å of ligand poses. Re-docking was performed in Glide using standard settings. The generated poses were ranked according to IFDScore, and we finally selected a pose with the lowest IFDScore as the interaction model. In order to validate our procedure, we applied it for the Hydroxy-PP – CBR1 complex. The result is shown in supplementary Fig. S1. The resulting model, i.e., the top-ranked pose, reproduced all interactions between Hydroxy-PP and CBR1 observed in the crystal structure. The positional and conformational RMSDs were 0.48 and 0.13 Å, respectively. These results suggested that our procedure is appropriate for producing reliable interaction models of **13h** as well as Hydroxy-PP. The docked model shown in Fig. 2 was generated using PyMOL (DeLano Scientific, San Carlos, CA, USA).

3.2.4. Cell culture experiments

BAEC were a generous gift from Dr. Junichi Nakagawa (Tokyo University of Agriculture, Abashiri, Japan). The cells were grown in Dulbecco's-modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified incubator containing 5% CO_2. In order to overexpress CBR1 in the cells, the cDNA for the enzyme was amplified by PCR from the bacterial expression vector[6] using primers (Supplementary Table S1). The amplified cDNA was subcloned at the *Eco*RI and *Sal*I sites into the mammalian pGW1 expression vector, and the sequence of the insert was verified by DNA sequencing. The pGW1 expression vector harboring the cDNA for CBR1 was transfected into BAEC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) when the cells were grown to 80% confluence in microplates. The empty vector was similarly transfected into the cells, which were used as control cells. The transfection efficacy was evaluated by Western blotting using the antibodies against CBR1[48] and human β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), of which β-actin was used as a loading control. After 48-h culture, the cells were washed twice with DMEM containing 2% fetal bovine serum and the antibiotics, and then treated for 24 h with 9,10-PQ. In the experiments with **13h** and **13p**, the cells were pretreated with or without the inhibitors 2 h before the treatment of 5 μM 9,10-PQ. Dimethylsulfoxide was the vehicle of the agents. The cell viability was evaluated by tetrazolium dye-based cytotoxicity assay using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium.[49] Data are expressed as means ± SD of at least three independent experiments. Statistical evaluation of the data was performed by using the unpaired Student's *t*-test and ANOVA followed by Fisher's test. A p value < 0.05 was considered statistically significant.

Conclusion

In this study, a series of 3-carboxamide derivatives of 8-hydroxy-2-iminochromene (**13a–13q**) and 8-hydroxycoumarin (**14a–14q**) were synthesized, and their inhibitory activities for CBR1 were evaluated. The structure-activity relationship of the synthesized

compounds revealed that the 8-hydroxyl and 2-imino groups on the chromene ring were essential to maintain the potent inhibitory effect. In addition, substituents in the 3-carboxamide chain of the 8-hydroxy-2-iminochromenes affected the inhibitory potency, and **13h** with 2-chlorophenyl ring as the substituent showed the lowest IC_{50} value, which is superior to those for the previously reported inhibitors of CBR1. **13h** also showed high selectivity to CBR1 over other SDRs (CBR3, DCXR and DHRS4) and AKRs (1B1, 1B10, 1C1, 1C2 and 1C4). Site-directed mutagenesis and molecular docking of **13h** in CBR1 demonstrated that the high inhibitory potency is attributed to the interactions of **13h** with the substrate-binding residues, Ser139, Met141, Tyr193 and Trp229, of which the interactions with Met141 and Trp229 contribute to the high selectivity to CBR1 over CBR3. We also provided a method that is useful to evaluate the efficacy of the inhibitor at a cellular level. These findings may contribute to future development of potent and selective CBR1 inhibitors.

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Abbreviations

AKR, aldo-keto reductase; AKR1B1; human aldose reductase; AKR1B10, human aldose reductase-like protein; BACE, bovine aorta endothelial cells; CBR, carbonyl reductase; DCXR, dicarbonyl/L-xylulose reductase; DHRS4, dehydrogenase/reductase SDR family member 4; Hydroxy-PP, 3-(1-*tert*-butyl-4-amino-1*H*-pyrazolo[3,4-d]pyrimidin-3-yl)phenol; 9,10-PQ, 9,10-phenanthrenequinone; SDR, short-chain dehydrogenase/reductase; *S*-tetralol, (*S*)-(+)-1,2,3,4-tetrahydro-1-naphthol.

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Legends of Scheme and Figures

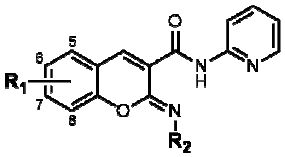
Scheme 1. Synthesis of chromene derivatives (**13a-13q** and **15**) and coumarin derivatives (**14a-14q**).

Fig. 1. Structures and inhibitory potency of known inhibitors of CBR1.

Fig. 2. **13h**-docked CBR1 model. $NADP^+$ (yellow) and residues (grey) within 4.0 Å from **13h** (pink) are depicted with possible H-bonds (dotted line) and their corresponding distances shown in Å. Only side-chains are shown for Ser139, Met141, Cys226, Tyr193, and Trp229.

Fig. 3. Inhibitory effects of **13h** and **13p** on 9,10-PQ cytotoxicity exacerbated by CBR1 overexpression in BAEC. (A) Dose-dependent effect of 9,10-PQ on viability of the cells transfected with CBR1 cDNA (●, cDNA) and control cells transfected with the vector alone (○, Vector). The overexpression of CBR1 in the transfected cells was analyzed by Western blotting (Inset). The cells were treated for 24 h with various concentrations of 9,10-PQ, and the cell viability was expressed as % ± S.D. (n=3) of control culture conditions. *Significant difference from the control cells, $p < 0.05$. (B) Effects of **13h** and **13p** on cell viability. The control and CBR1-overexpressing cells were pretreated for 2 h with the vehicle alone (0), **13h** and **13p** (4 and 10 μM), and then treated for 24 h with 5 μM 9,10-PQ. The viability of the control cells pretreated with the vehicle is taken as 100%. *Significant difference from the CBR1-overexpressing cells pretreated with vehicle alone (0) inhibitors, $p < 0.05$.

Table 1. Inhibitory effects of the chromene derivatives with or without 4-methoxyphenyl moiety on CBR1, AKR1B10 and AKR1B1



Entry.	R_1	R_2	IC_{50} (μM)		
			CBR1	AKR1B10	AKR1B1
1	7-OH	4-Methoxyphenyl	0.25 ± 0.021	0.006 [a]	0.011 [a]
2	5-OH	4-Methoxyphenyl	0.53 ± 0.062	0.29 [a]	> 10 [b]
3	6-OH	4-Methoxyphenyl	> 10 [b]	> 10 [b]	> 10 [b]
4	8-OH	4-Methoxyphenyl	> 10 [b]	> 10 [b]	> 10 [b]
5	H	4-Methoxyphenyl	> 10 [b]	> 10 [b]	> 10 [b]
6	7-OH	H	3.0 ± 0.27	0.099 ± 0.010	0.52 ± 0.078
7	5-OH	H	5.3 ± 1.1	1.5 ± 0.045	> 10 [b]
8	6-OH	H	> 10 [b]	> 10 [b]	> 10 [b]
9	8-OH	H	0.32 ± 0.0070	> 10 [b]	> 10 [b]

[a] The values are taken from Refs. 31 and 32.

[b] Less than 33% inhibition at 10 μM.

Table 2. Inhibition of CBR1 by 2-carboxamide derivatives of 8-hydroxy-2-iminochromene and 8-hydroxycoumarin

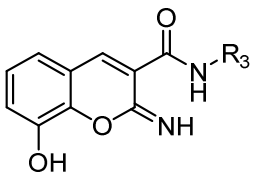
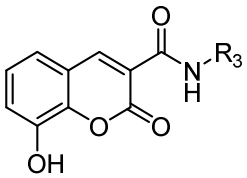
R_3				
	Entry	IC_{50} (μM)	Entry	IC_{50} (μM)
Phenyl	**13a**	0.21 ± 0.012	**14a**	1.9 ± 0.16
2-Hydroxyphenyl	**13b**	0.33 ± 0.025	**14b**	0.47 ± 0.028
3-Hydroxyphenyl	**13c**	0.15 ± 0.011	**14c**	0.37 ± 0.030
4-Hydroxyphenyl	**13d**	0.88 ± 0.045	**14d**	1.3 ± 0.066
2-Fluorophenyl	**13e**	0.31 ± 0.037	**14e**	1.8 ± 0.42
3-Fluorophenyl	**13f**	0.37 ± 0.045	**14f**	2.5 ± 0.15
4-Fluorophenyl	**13g**	0.44 ± 0.086	**14g**	2.5 ± 0.11
2-Chlorophenyl	**13h**	0.034 ± 0.0035	**14h**	0.26 ± 0.037
3-Chlorophenyl	**13i**	0.12 ± 0.015	**14i**	1.5 ± 0.082
4-Chlorophenyl	**13j**	0.22 ± 0.015	**14j**	0.45 ± 0.0082
Benzyl	**13k**	0.33 ± 0.03	**14k**	0.92 ± 0.0091
2-Hydroxybenzyl	**13l**	0.35 ± 0.026	**14l**	1.3 ± 0.18
3-Hydroxybenzyl	**13m**	0.11 ± 0.0011	**14m**	1.1 ± 0.051
4-Hydroxybenzyl	**13n**	0.17 ± 0.022	**14n**	0.82 ± 0.013
2-Chlorobenzyl	**13o**	0.10 ± 0.013	**14o**	0.41 ± 0.019
3-Chlorobenzyl	**13p**	0.090 ± 0.00064	**14p**	1.1 ± 0.072
4-Chlorobenzyl	**13q**	0.26 ± 0.0072	**14q**	1.0 ± 0.0011

Table 3. Inhibitory effects of 13h, 13p and 13o on human enzymes in the SDR and AKR superfamilies.

Enzyme	13h		13p		13o		Luteolin	
	IC_{50} [a]	SR [b]	IC_{50} [a]	SR [b]	IC_{50} [a]	SR [b]	IC_{50} [a]	SR [b]
CBR1	0.034	-	0.090	-	0.10	-	0.095 [c]	-
CBR3	1.5±0.16	44	>10	>110	2.5±0.026	25	0.53±0.052	6
DCXR	>10	>290	>10	>110	>10	>100	6.6±0.39	69
DHRS4	>10	>290	>10	>110	>10	>100	5.3±0.38	56
AKR1B10	>10	>290	3.7±0.010	34	4.8±0.31	48	0.73±0.058	8
AKR1B1	>10	>290	>10	>110	>10	>100	0.18±0.010	2
AKR1C1	>10	>290	>10	>110	>10	>100	1.9±0.11	20
AKR1C2	>10	>290	>10	>110	>10	>100	6.7±0.31	71
AKR1C4	>10	>290	>10	>110	>10	>100	1.7±0.10	18

[a] IC_{50} (μM) was determined as described in the section 3.2.2.
>10: less than 36% inhibition at 10 μM.

[b] SR: selectivity ratio of the IC_{50} value for other enzyme to that for CBR1.

[c] The value is taken from Ref. 27.

Table 4. Effects of mutations of CBR1 on the K_i values of 12h and 12p

Enzyme	13h		13p	
	K_i (nM) [a]	Mu/Wt [b]	K_i (nM) [a]	Mu/Wt [b]
Wild type	15±1.9	-	47±2.9	-
Met141Gln	180±17	12	530±21	11
Met141Val	47±2.1	3	97±8.1	2
Trp229Phe	69±8.5	5	110±5.3	2
Trp229Leu	2400±330	160	1600±160	34

[a] The inhibition patterns in the $NADP^+$-linked *S*-tetralol dehydrogenase activity were all competitive with respect to the substrate.

[b] Mu/Wt represents the ratio of the K_i value for the mutant enzyme to that for the wild type enzyme.