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Human constitutive androstane receptor agonist DL5016: a novel sensitizer for cyclophosphamide-based chemotherapies

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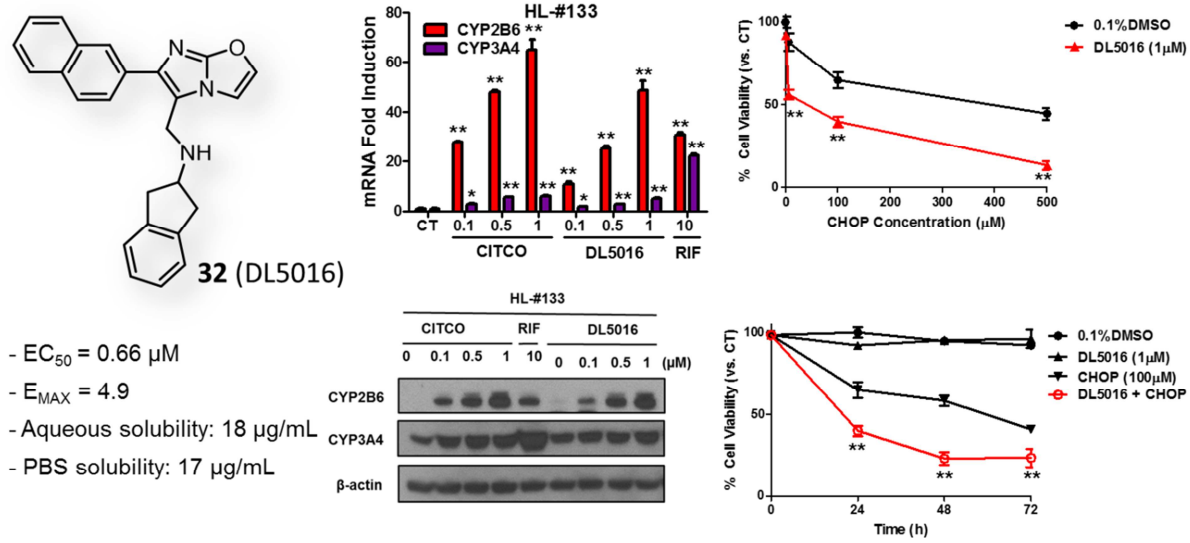
KEYWORDS: Human constitutive androstane receptor, cyclophosphamide, structure-activity relationship, co-culture, chemotherapy

ABBREVIATIONS

CAR, constitutive androstane receptor; CPA, Cyclophosphamide; CYP2B6, cytochrome P450 2B6; CYP3A4, cytochrome P450 3A4; CLL, chronic lymphocytic leukemia; ALL, lymphoblastic leukemia; PXR, pregnane X receptor; TCPOBOP, 1,4-bis(3,5-dichloro-2-pyridinyl oxy)benzene; HTS, high throughput screening; CITCO, 6-(4-chlorophenyl)imidazo-[2,1-*b*]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl)oxime; LBD, ligand binding domain; TLC, thin-layer chromatography; RIF, rifampicin; PB, phenobarbital; HPH, human primary hepatocytes.

GRAPHIC ABSTRACT



- EC_{50} = 0.66 μM
- E_{MAX} = 4.9
- Aqueous solubility: 18 μg/mL
- PBS solubility: 17 μg/mL

ABSTRACT

The DNA alkylating prodrug cyclophosphamide (CPA), alone or in combination with other agents, is one of the most commonly used anti-cancer agents. As a prodrug, CPA is activated by cytochrome P450 2B6 (CYP2B6), which is transcriptionally regulated by the human constitutive androstane receptor (hCAR). Therefore, hCAR agonists represent novel sensitizers for CPA-based therapies. Among known hCAR agonists, compound 6-(4-chlorophenyl)imidazo-[2,1-*b*]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl)oxime (CITCO) is the most potent and broadly utilized in biological studies. Through structural modification of CITCO, we have developed a novel compound DL5016 (**32**), which has an EC_{50} value of 0.66 μM and E_{MAX} value of 4.9 when activating hCAR. DL5016 robustly induced the expression of hCAR target genes CYP2B6, at both the mRNA and protein levels, and caused translocation of hCAR from the cytoplasm to the nucleus in human primary hepatocytes. The effects of DL5016 were highlighted by dramatically enhancing the efficacy of CPA-based cytotoxicity to non-Hodgkin lymphoma cells.

1. Introduction

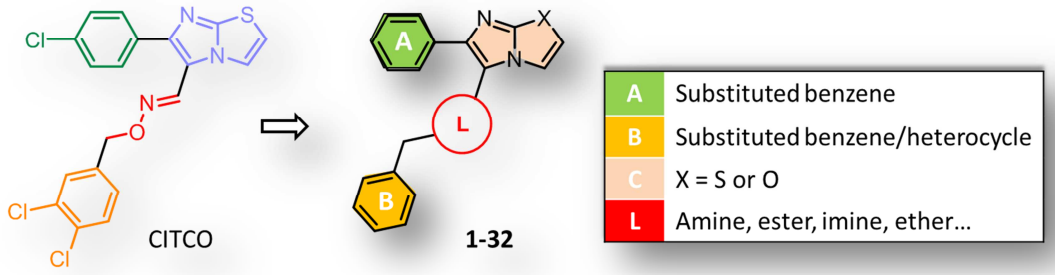
Cyclophosphamide (CPA), an alkylating prodrug of the oxazaphosphorine family, remains one of the most widely prescribed anti-cancer drugs [1]. CPA is used, often in combination with other agents, for the treatment of numerous malignant diseases including Hodgkin and non-Hodgkin lymphomas, multiple myeloma, chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), and solid tumors such as ovarian cancer, breast cancer, small cell lung cancer, and sarcoma [1]. Although CPA and its combination regimens have successfully improved the survival rates of patients with early stage cancer, their effects for those with late stage diseases are often limited. Thus, there is still an urgent need for new CPA-based treatments with an enhanced efficacy and better controlled toxicity. One of the potential solutions is to increase the metabolic conversion of CPA to its pharmacologically active metabolite [2].

CPA is biotransformed predominantly in the liver to the active metabolite 4-hydroxy cyclophosphamide by cytochrome P450 isozyme 2B6 (CYP2B6) [3,4]. In addition to this desirable metabolic pathway, CPA can also be converted into a pharmacologically inactive metabolite dechloroethyl-CPA, along with another toxic metabolite, 2-chloro acetaldehyde, almost exclusively by CYP3A4 [5]. It is well documented that in the human body, the expression of CYP2B6 and CYP3A4 are positively regulated by nuclear receptors constitutive androstane receptor (CAR) and pregnane X receptor (PXR), respectively, with certain levels of cross-talk [6-9]. While activation of PXR has been shown to induce expression of CYP3A4 and CYP2B6 in concert, selective activation of CAR has shown preferential induction of CYP2B6 over CYP3A4 [6-9]. Therefore, development of potent and selective human CAR (hCAR) agonists represents a promising approach to sensitize CPA-based chemotherapies [2].

As a part of its xenobiotic response mechanism, CAR is activated by a wide variety of ligands including environmental chemicals and drugs [10-12]. For instance, Tzameli and coworkers showed that the cytochrome P450 inducing agent 1,4-bis(3,5-dichloro-2-pyridinyl oxy)benzene (TCPOBOP) [13] functions by selective activation of mouse CAR [14]. Sueyoshi and coworkers reported that the polybrominated diphenyl ether, BDE-47, was a non-selective activator for both CAR and PXR [15]. Various high-throughput screening (HTS) efforts have been devoted to the identification of novel and selective hCAR agonists [16-19]. Among others, a screening campaign from GlaxoSmithKline led to the identification of 6-(4-chlorophenyl)imidazo-[2,1-*b*]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl)oxime (CITCO, Figure 1), which binds to the ligand binding domain (LBD) of hCAR and activates the receptor with a high potency [20,21]. Since its discovery, CITCO has been widely used as a biological tool in numerous studies investigating the functions of hCAR [22-25]. Although CITCO can activate human PXR at higher concentrations, its high affinity for hCAR leads to the potent and preferred induction of CYP2B6 over CYP3A4 in human primary hepatocytes (HPH) [20]. Recently, synthetic efforts have started to emerge in developing novel hCAR agonists. Smutny and coworkers disclosed their efforts in the synthesis of 2-(3-methoxyphenyl) quinazoline-based hCAR agonists, although the top compounds of the series failed to significantly improve the expression levels of hCAR regulated genes [26].

Herein, we disclose the development of a novel hCAR agonist, DL5016, from a series of new compounds **1**-**32** (Figure 1). The compounds have been designed by modifying the chemical structure of CITCO. Specifically, the oxime linker of CITCO, which might cause chemical instability [26,27], was replaced by functional groups such as amine, amide, imine, and ether. In addition, the three hydrophobic ring systems of CITCO, imidazothiazole, 3,4-diCl-phenyl and 4-

Cl-phenyl, were optimized using a variety of substituted arene moieties, for their hCAR potency and selectivity over hPXR. Our lead compound of the series, DL5016, was stable in an aqueous environment and, activated hCAR with good potency and selectivity over hPXR. DL5016 caused hCAR translocation from the cytoplasm into the nucleus of HPH and induced the expression of CYP2B6 at both the mRNA and protein levels. In co-culture of HPH and human non-Hodgkin lymphoma cells, DL5016 also potentiated the cytotoxicity of the CPA-based combination regimen CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) against



the targeted lymphoma cells.

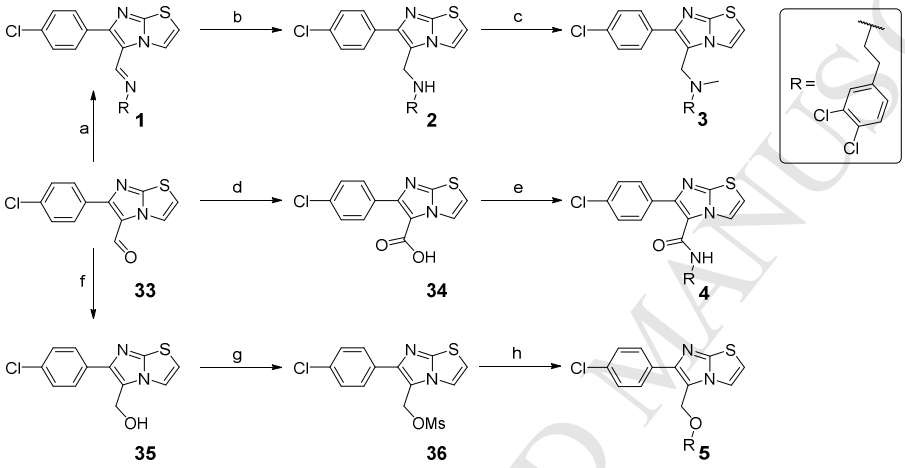
Figure 1. Design of compounds **1-32** by modifying the structure of CITCO.

2. Results and discussion

2.1. Chemistry

The syntheses of compounds **1-32** are summarized in Schemes 1-5. To optimize the linker of the agonist, compounds **1-5** (Scheme 1) were prepared using a common precursor **33** (Scheme S1). Condensation of aldehyde **33** with 2-(3,4-dichloro phenyl)ethan-1-amine in the presence of acetic acid (AcOH) generated imine **1** in modest yields. Reduction of compound **1** using $NaBH_4$ gave secondary amine **2** in modest yields. Methylation of the amino group of compound **2** using MeI provided tertiary amine **3** in modest yields [28]. The amide analog **4** was synthesized via a

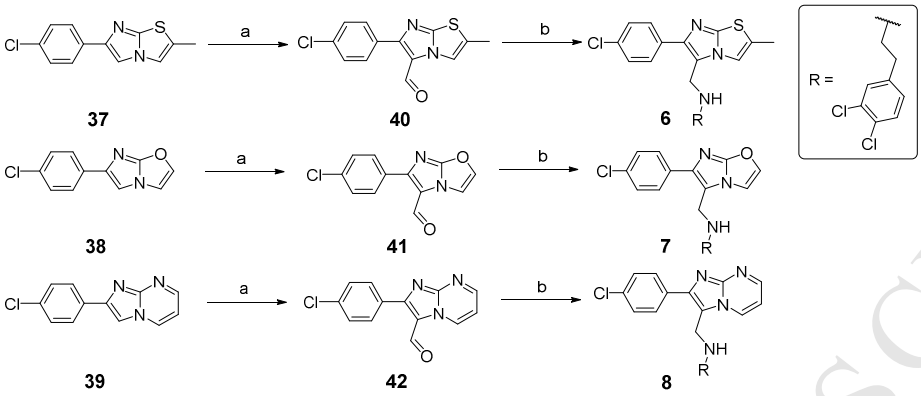
two-step procedure from **33**. $NaClO_2$ mediated oxidation of aldehyde **33** generated carboxylic acid **34**, which was coupled to 2-(3,4-dichlorophenyl)ethan-1-amine using EDC and DMAP to generate amide **4** [29]. To get the ether analog **5**, aldehyde **33** was first reduced to primary alcohol **35** using $NaBH_4$ in good yields. Then, the resulting hydroxy group of **35** was activated using MsCl to give compound **36** [30]. Finally, nucleophilic substitution of mesylate **36** using 2-(3,4-dichlorophenyl)ethan-1-ol as a nucleophile provided ether analog **5** [31].



Scheme 1. Synthesis of compounds **1-5**. Reagents and conditions: (a) 2-(3,4-dichlorophenyl)ethan-1-amine, AcOH, EtOH, 90 ^oC, overnight, 50%; (b) $NaBH_4$, MeOH, rt, 6 h, 64%; (c) MeI, NaH, DMF, 60 ^oC, overnight, 61%, (d) $NaClO_2$, NaH_2PO_4, 2-methyl-2-butene, *t*-BuOH/H_2O/THF, rt, overnight, 85%; (e) 2-(3,4-dichlorophenyl)ethan-1-amine, EDCI, DMAP, DCM, rt, overnight, 65%; (f) $NaBH_4$, MeOH, rt, 2 h, 75%; (g) MsCl, Et_3N, DCM, 2 h; (h) 2-(3,4-dichlorophenyl) ethan-1-ol, K_2CO_3, DMF, 60 ^oC, overnight, 28% for two steps.

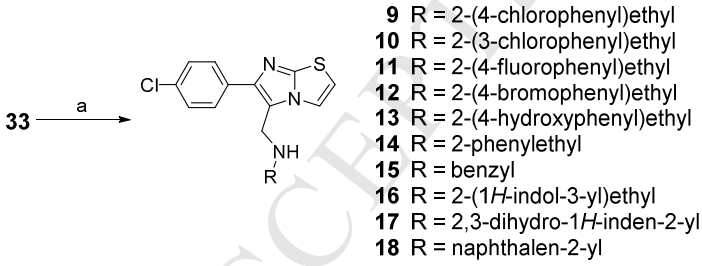
To optimize the imidazothiazole core of CITCO, three compounds (**6**-**8**) were synthesized using imidazole derivatives **37**-**39** as the starting materials (Scheme 2). Vilsmeier reaction of compounds **37**-**39** using DMF and $POCl_3$ gave aldehydes **40**-**42** in modest to good yields [32].

Reductive amination of aldehydes **40-42** using 2-(3,4-dichloro phenyl)ethan-1-amine give final compounds **6-8** in modest yields [33].



Scheme 2. Synthesis of compounds **6-8**. Reagents and conditions: (a) DMF, $POCl_3$, $CHCl_3$, 0 ^oC to reflux, 6 h, 65-87%; (b) i) 2-(3,4-dichlorophenyl)ethan-1-amine, AcOH, EtOH, reflux, overnight; ii) $NaBH_4$, MeOH, rt, overnight, 22-57% for two steps.

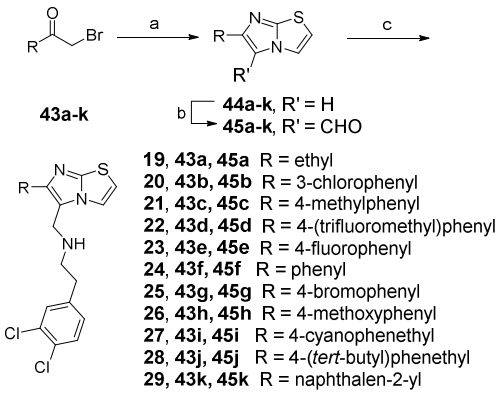
To optimize B-ring, compounds **9-18** were synthesized (Scheme 3). Condensation of various amines with aldehyde **33** generated the corresponding imines, which were then submitted to a $NaBH_4$ mediated reduction to give the final compounds **9-18** in modest overall yields [33].



Scheme 3. Synthesis of compounds **9-18**. Reagents and conditions: (a) i) R-NH_2, AcOH, EtOH, reflux, overnight; ii) $NaBH_4$, MeOH, rt, overnight, 15-49% for two steps.

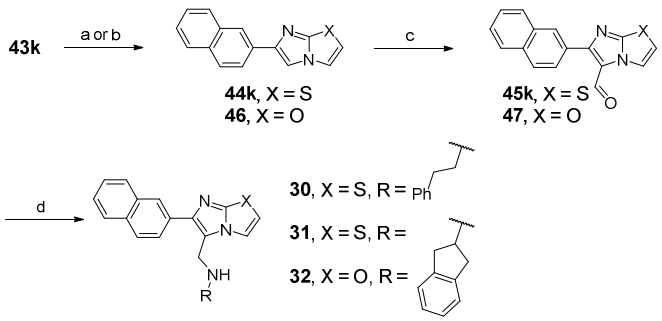
To optimize A-ring, compounds **19-29** were synthesized using a collection of bromomethyl ketones **43a-k** (Scheme 4). Treatment of compounds **43a-k** with thiazol-2-amine in refluxing EtOH provided imidazothiazoles **44a-k** [34], which were submitted to Vilsmeier reaction using

DMF and $POCl_3$ to give aldehydes **45a-k** in good yields [32]. Reductive amination of aldehydes **45a-k** with 2-(3,4-dichlorophenyl)ethan-1- amine generated compounds **19-29** in good overall yields [33].



Scheme 4. Synthesis of compounds **19**-**29**. Reagents and conditions: (a) thiazol-2-amine, EtOH, reflux, overnight; (b) DMF, $POCl_3$, $CHCl_3$, 0 ^oC to reflux, 6 h, 51-69% for two steps; (c) i) 2-(3,4-dichlorophenyl)ethan-1- amine, AcOH, EtOH, reflux, overnight; ii) $NaBH_4$, MeOH, rt, overnight, 12-56% for two steps.

The syntheses of compounds **30**-**32** started with bromomethyl ketone **43k** (Scheme 5). Condensation of compound **43k** with either 2-amino-thiazole or 2-amino-oxazole gave compounds **44k** and **46**, respectively [34,35]. Vilsmeier reaction of compounds **44k** and **46** using DMF and $POCl_3$ yielded the corresponding aldehydes **45k** and **47** [32], which underwent reductive amination with either 2-phenylethan-1-amine or 2,3-dihydro-1*H*- inden-2-amine to provide the final products **30**-**32** [33].



Scheme 5. Synthesis of compounds **30**-**32**. Reagents and conditions: (a) 2-amino-thiazole, EtOH, reflux, overnight; (b) i) 2-amino-oxazole, CH_3CN/THF, rt, 24 h; ii) $TiCl_4$, toluene; 0-100 ^oC, 4 h; (c) DMF, $POCl_3$, $CHCl_3$, 0 ^oC to reflux, 6 h, 51-79% for two steps; (d) i) amine, AcOH, EtOH, reflux, overnight; ii) $NaBH_4$, MeOH, 0-rt, overnight, 26-42% for two steps.

2.2. Structure-activity relationship (SAR)

To modify the chemical structure of CITCO, we first replaced the unstable oxime linker with various functional groups (**1**-**5**, Figure 2A, blue). The EC_{50} and E_{MAX} values of new compounds were determined using an hCAR luciferase gene reporter assay [18]. Compared to the parent CITCO, the imine analog **1** was a much weaker agonist for hCAR (EC_{50} = 54 µM vs 3.1 µM for CITCO, Figure 2A). The poor potency of this compound was probably due to the instability of the imine group, which hydrolyzes readily in aqueous solutions to generate the inactive intermediate **33**. The secondary amine **2**, on the other hand, provided a 1.4-fold increase in potency and a slightly decreased E_{MAX} value compared to CITCO. The additional methyl group of the tertiary amine analog **3** turned out to be detrimental to both the EC_{50} and E_{MAX} values of the compound. Decreased activities were also observed for compound **4** that employed a more rigid amide linker. Interestingly, the ether analog **5** indicated an excellent EC_{50} value of 0.19 µM while having a slightly decreased E_{MAX} value. These results showed that flexible small linkers, such as secondary amine and ether, are preferred for hCAR agonist activity.

Using secondary amine **2** as a template, three compounds **6**-**8** were synthesized by modifying the imidazothiazole core (Figure 2A, red). Introduction of a methyl group at the 2-position of the thiazole ring caused a 5.5-fold (EC_{50} = 2.2 μM vs 0.4 μM for **2**) drop of the potency. This result is consistent with the fact that the imidazothiazole moiety of CITCO binds to the bottom of a hydrophobic pocket in the LBD of hCAR (Figure 6) [21]. Replacement of the sulfur atom with an oxygen yielded compound **7** with a 3.2-fold decrease in the EC_{50} value. However, compared to compound **2**, the resulting compound also demonstrated a small increase in the E_{MAX} value. These results showed that the imidazo[2,1-*b*]oxazole could be a good substitution of the imidazothiazole core, which forms toxic metabolites *in vivo* [36]. Expansion of the core to an imidazo[1,2-*a*]pyrimidine ring system resulted in a new compound (**8**) with a 2-fold decrease in potency and a slight decrease of the E_{MAX} value.

Compounds **2**, **5**, **7** and **8** were selected for further evaluation based on their effects in activating hCAR and hPXR using the HepG2 cell reporter assays (Figure 2B) [37]. At a concentration of 1 μM, compounds **2** and **5** demonstrated similar efficacies in activating hCAR, although the more potent compound **5** in the hCAR agonist assay also significantly activated hPXR. These results together indicated that the secondary amino linker is key for both potency and specificity of hCAR agonists. Note that the imidazo[2,1-*b*]oxazole analog **7**, although indicated a slight decrease in hCAR activation potency, still indicated good specificity for hCAR. The imidazo[1,2-*a*]pyrimidine analog **8**, at the same tested concentration (1 μM), was less potent compared to CITCO and the other three compounds.

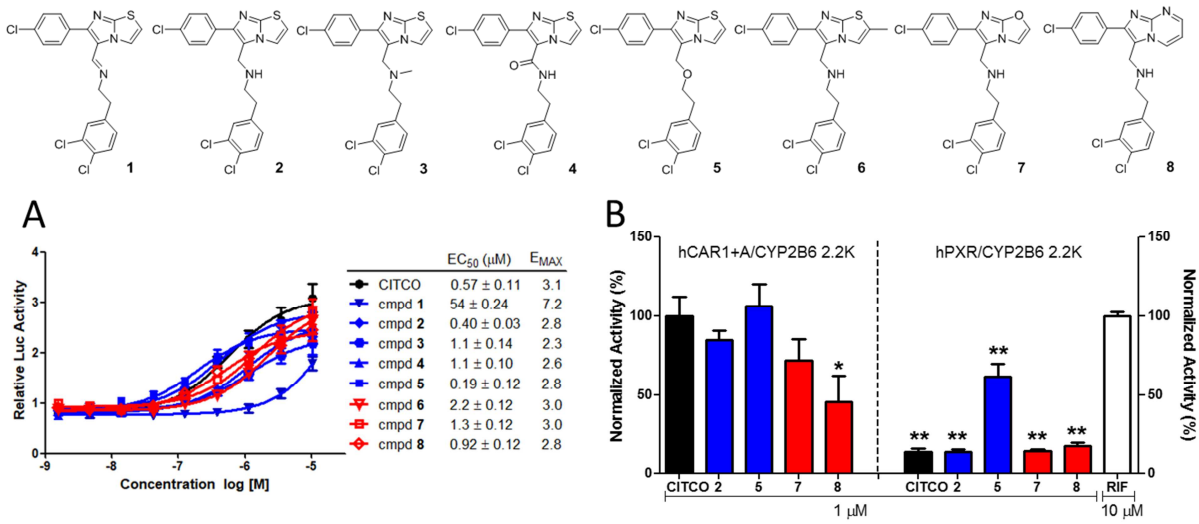


Figure 2. Activation of hCAR and hPXR by CITCO and compounds **1**-**8**. (A) Concentration dependent activation curves along with EC_{50} and E_{MAX} values of CITCO and compounds **1**-**8** in the hCAR agonist luciferase gene reporter assays. EC_{50} values obtained from at least three independent experiments in quadruplicate were expressed as mean ± SEM. E_{MAX} values were calculated by nonlinear regression. (B) Activation of hCAR and hPXR by CITCO and compounds **2**, **5**, **7** and **8** in the HepG2 cell reporter assays. Data obtained from three independent experiments were expressed as mean ± SD normalized against positive control CITCO for hCAR activities or rifampicin (RIF) for hPXR activities. *, $P < 0.05$; **, $P < 0.01$.

Next, substitutions of the 3,4-diCl-phenyl ring of CITCO were studied (Figure 3). When one of the two Cl atoms was removed, the resulting compounds **9** and **10** demonstrated 1.6- and 7-fold decreases in potency, respectively (Figure 3A). These results indicated that the 4-Cl group contributes more significantly to hCAR agonist activity. When the 4-Cl was changed to a smaller F, the resulting compound **11** indicated a 3-fold decrease of potency while keeping a similar E_{MAX} value. When a larger Br (**12**) was introduced to the same position, although the EC_{50} was further decreased, the E_{MAX} value of the resulting compound increased significantly.

Inclusion of a polar OH group led to a new compound (**13**) with a significantly decreased EC_{50}, indicating that polar substitutions were not preferred on the bottom ring. Interestingly, the analog with an unsubstituted phenyl group (**14**) turned out to be a potent hCAR agonist, and its analog with one CH_2 shorter (**15**) indicated an over 11-fold decrease in potency. These results highlighted the importance of linker length in maintaining the agonistic potency of hCAR ligands. Three new compounds (**16**-**18**) with fused ring systems were also tested. While the indole analog **16** generated modest potency (EC_{50} = 2.7 μM) with a significant increase in E_{MAX}, it was exciting to see the 2,3-dihydro-1*H*-inden-2-yl analog **17** indicated good EC_{50} and E_{MAX} values. The large naphthyl compound (**18**) was inactive towards hCAR.

Compounds **9**, **14,** and **17** were selected for further evaluation of their activities in activating hCAR and hPXR using the HepG2 cell reporter assays (Figure 3B) [37]. All three compounds, at 1 μM, had similar activation effects compared to CITCO, while also maintaining selectivity for hCAR against hPXR.

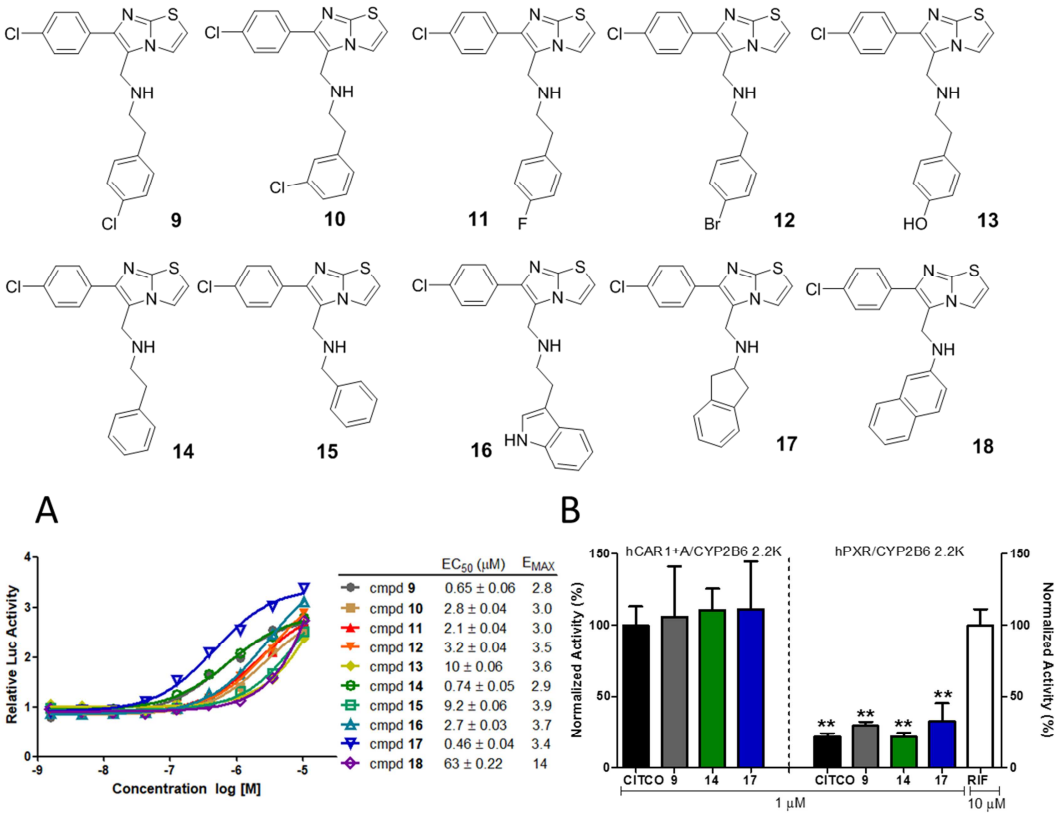


Figure 3. Activation of hCAR and hPXR by CITCO and compounds **9**-**18**. (A) Concentration dependent activation curves alongside EC_{50} and E_{MAX} values of compounds **9**-**18** in high throughput hCAR agonist luciferase gene reporter assays. EC_{50} values obtained from at least three independent experiments in quadruplicate were expressed as mean ± SEM. E_{MAX} values were calculated by nonlinear regression. (B) Activation of hCAR and hPXR by CITCO and compounds **9**, **14**, and **17** in the HepG2 cell reporter assays. Data obtained from three independent experiments were expressed as mean ± SD normalized against the positive control, CITCO for hCAR activities or RIF for hPXR activities. **, $P < 0.01$.

We then optimized the 4-Cl-phenyl ring of CITCO using a collection of compounds **19**-**29** (Figure 4). Replacement of the phenyl group with a small aliphatic ethyl group (**19**) caused a dramatic drop in hCAR activity (Figure 4A). This result indicated that an aromatic system at this

position is important for the potency of hCAR agonists. Moving the Cl from the 4-position to 3-position (**20**) caused a 2.3-fold decrease in the EC_{50} (0.93 μM vs 0.40 μM) for compound **2**. However, the new compound (**20**) also had an increased E_{MAX} (from 2.8 to 3.2). These results suggested that the 3-substitution might also be beneficial for new agonists. Replacement of the Cl with a similar-sized Me group led to the formation of compound **21** that had similar EC_{50} and E_{MAX} values, while with a more polar CF_3 group, new compound **22** was a much weaker agonist. Substitution of the Cl with smaller F or H atoms led to new compounds **23-24** with excellent potencies, although their E_{MAX} values were decreased. The interesting profiles of these compounds (**23-24**) make them good candidates for hCAR activation at low concentrations. In addition, large substitution at the 4-position, such as Br (**25**), OMe (**26**), CN (**27**) and *t*-Bu (**28**), were also tested. While compounds **25** and **26** had comparable EC_{50} and E_{MAX} values to the parent **2**, compounds **27** and **28** were significantly less active. Interestingly, the naphthyl analog **29**, although with a 3-fold decrease in potency, had a large E_{MAX} value of 3.6. These results indicated that the naphthyl group at this position is also preferred for hCAR agonists.

The promising compounds **23**, **24**, and **29** were then selected and further evaluated for their activation effects, of hCAR and hPXR, using the HepG2 cell reporter assays (Figure 4B) [37]. While compounds **23** and **24** indicated slightly decreased activation potency, compared with CITCO, the naphthyl analog **29** showed a slight increase in activation of hCAR. At the same time, compound **29** retained selectivity for hCAR over hPXR.

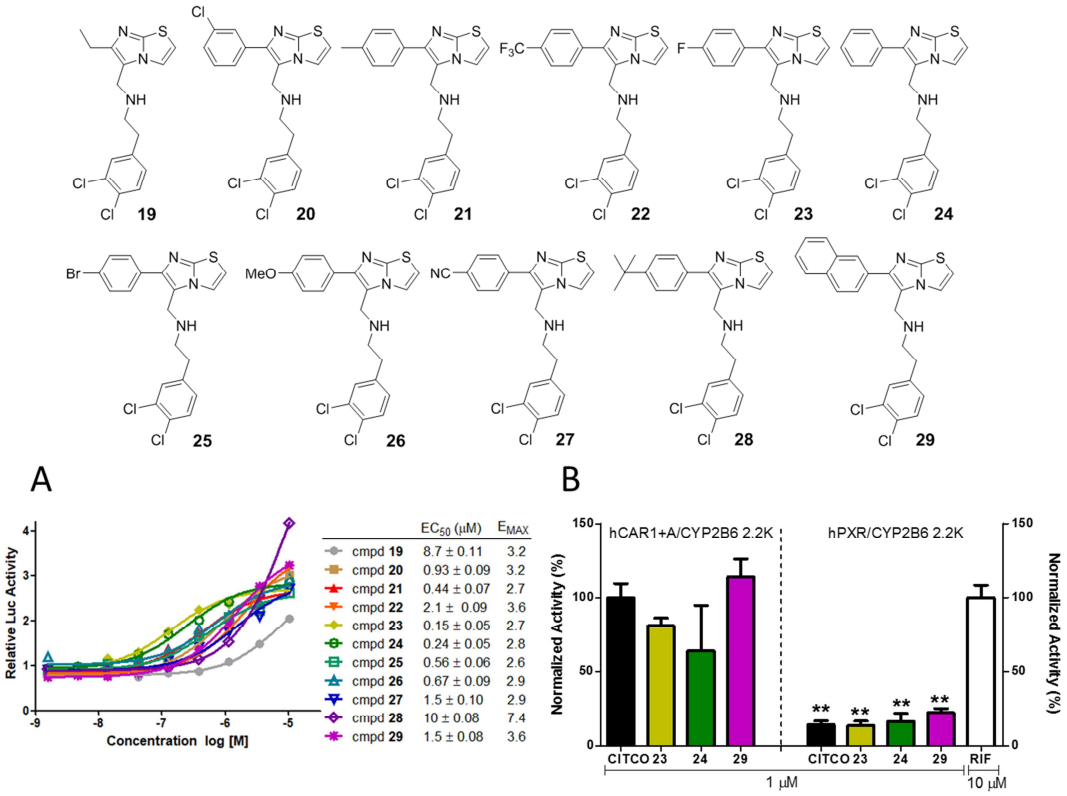


Figure 4. Activation of hCAR and hPXR by CITCO and compounds **19**-**29**. (A) Concentration dependent activation curves alongside EC_{50} and E_{MAX} values of compounds **19**-**29** in high-throughput hCAR agonist luciferase gene reporter assays. EC_{50} values obtained from at least three independent experiments in quadruplicate were expressed as mean ± SEM. E_{MAX} values were calculated by nonlinear regression. (B) Activation of hCAR and hPXR by CITCO and compounds **23**, **24**, and **29** in the HepG2 cell reporter assays. Data obtained from three independent experiments were expressed as mean ± SD normalized against the positive control, CITCO for hCAR activities or RIF for hPXR activities. **, $P < 0.01$.

Finally, we combined the desirable features from the above compounds **1**-**29** and synthesized compounds **30**-**32** (Figure 5). While compounds **30** and **31** had similar activities, compound **32** (DL5016) showed excellent EC_{50} and E_{MAX} values (Figure 5A). These three compounds were also evaluated in the HepG2 cell reporter assays and compared to CITCO (Figure 5B) [37].

Compound **30** caused similar hCAR activation with respect to CITCO; however, it also significantly activated hPXR. Compound **31** demonstrated enhanced hCAR activation over CITCO, while hPXR activation was also slightly increased. It was exciting to see that compound DL5016 demonstrated significant improvement in hCAR activation over CITCO while maintaining selectivity over hPXR.

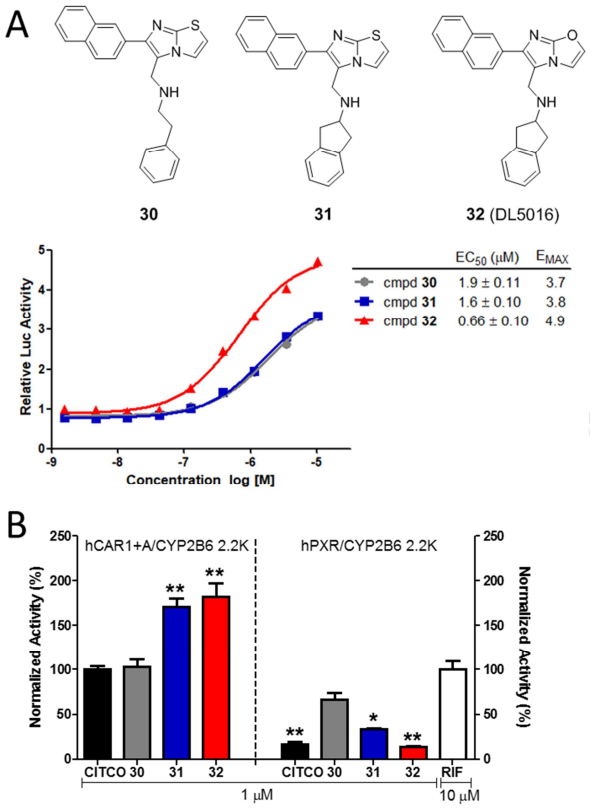


Figure 5. Activation of hCAR and hPXR by CITCO and compounds **30**-**32**. (A) Concentration dependent activation curves alongside EC_{50} and E_{MAX} values of compounds **30**-**32** in high-throughput hCAR agonist luciferase gene reporter assays. EC_{50} values obtained from at least three independent experiments in quadruplicate were expressed as mean ± SEM. E_{MAX} values were calculated by nonlinear regression. (B) Activation of hCAR and hPXR by CITCO and

compounds **30**-**32** in the HepG2 cell reporter assays. Data obtained from three independent experiments were expressed as mean ± SD normalized against the positive control, CITCO for hCAR activities or RIF for hPXR activities. *, $P < 0.05$; **, $P < 0.01$.

2.3. Binding mode of DL5016 in the LBD of hCAR

Compound DL5016 indicated an overall binding mode similar to that of CITCO (Figure 6) [21]. Specifically, the 2,3-dihydro-1*H*-inden-2-yl fragment of DL5016 occupied the same hydrophobic pocket (Tyr224, Phe132, and Phe217) as the dichlorophenyl group of CITCO. Because the length of 2,3-dihydro-1*H*-inden-2-yl is slightly shorter than the dichlorophenyl group, it fits deeper into this hydrophobic pocket than dichlorophenyl, which might explain why the 2,3-dihydro-1*H*-inden-2-yl analog demonstrated improved agonistic activity compared to the corresponding dichlorophenyl analog (**17** vs **2**). Similar to the imidazothiazole core of CITCO, the imidazo[2,1-*b*]oxazole core of DL5016, located at the junction of helixes 3 and 5 in close proximity to Met168 (H3) and Cys202 (H5). This explains why an additional methyl group on the imidazothiazole core was not tolerated (**6** vs **2**). The S atom in the imidazothiazole core of CITCO is 3.0 Å away from the side chain hydroxyl group of Tyr326, implying a possible H-bonding interaction. Considering the fact that Tyr326 belongs to helix 10 which is adjacent to helix X and AF2, this interaction might contribute to the agonistic activity of CITCO with respect to hCAR. The distance between the imidazothiazole oxygen atom of DL5016 to the same Tyr326 residue was 4.4 Å, which could explain the slightly decreased EC_{50} value of DL5016 compared to CITCO. The naphthyl group of compound DL5016, similar to the p-Cl-phenyl group of CITCO, pointed directly to the residue Gly229 of helix 6 and Leu239 in helix 7. This result explains why large substitutions could not be tolerated on the A-ring in our SAR studies. It is worth noting that the naphthyl group formed a favorable π-π stacking interaction

with the side chain of Phe234. The same interaction was not observed in the crystal structure of CITCO in hCAR.

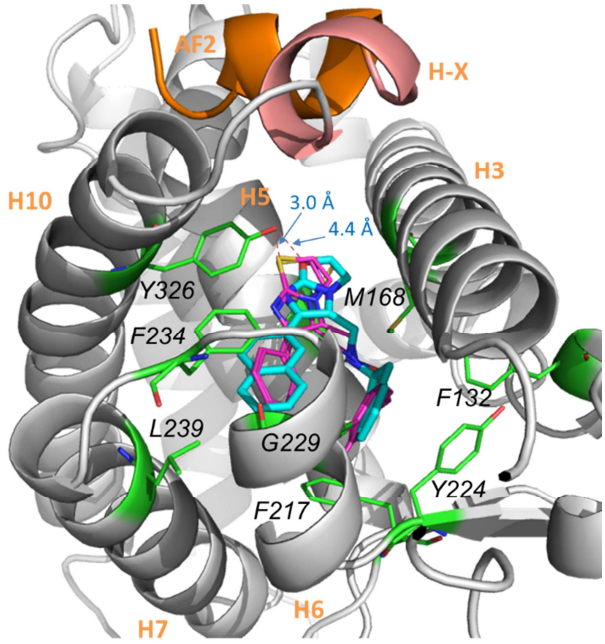


Figure 6. Docking result of compound DL5016 (cyan) in the LBD of hCAR (grey) in comparison with CITCO (magenta, PDB: 1XVP). Key residues of hCAR that interact with CITCO and DL5016 are highlighted in green, the helix X (H-X) is shown in light pink, and the activation function 2 helix (AF2) is orange.

2.4. Physiochemical properties of DL5016

Compound DL5016 demonstrated similar hCAR agonist potency and an improved E_{MAX} value compared to CITCO (Table 1). Unlike CITCO, which gave unstable results from the hCAR luciferase gene reporter assay [26,27], compound DL5016 indicated excellent stability. In addition, the potential toxicophores of CITCO, the thiazole and oxime groups, were replaced in compound DL5016 by oxazole and amino groups, respectively. Moreover, CITCO is a highly hydrophobic (logP 7.2) structure without an ionizable functional group, while compound

DL5016 indicated a significantly decreased logP value and an ionizable amino group (pK_a 7.85). The solubilities of compound DL5016 in various solvent systems have also been tested. DL5016 indicated reasonable aqueous solubility in both H_2O and PBS buffer.

Table 1
Comparison of properties for CITCO and DL5016.

		CITCO	DL5016
EC_{50}		0.57 ± 0.11[a]	0.66 ± 0.10
E_{MAX}		3.1[a]	4.9
E_{MAX} / EC_{50}		5.4	7.2
Potential toxicophore [38]		thiazole, oxime	none
logP		7.26	4.62
pK_a		NA	7.85
Solubility (μg/mL)	H_2O	ND[b]	18 ± 5
	PBS buffer	ND[b]	17 ± 5

[a]Number shown here is an average of six different batches of samples.
[b]The solubility was not determined.

2.5. Activation of hCAR induces hepatic expression of CYP2B6 and CYP3A4

Numerous examples have shown that compounds identified using hCAR gene reporter assays might not be able to induce the expression of the CYP2B6 gene, the rate-determining enzyme for CPA bioactivation [26,39]. To study the effects of compound DL5016 on the expression of CPA-metabolizing enzymes, HPH were treated with CITCO, DL5016, and the known hPXR activator Rifampin (RIF, Figure 7). For HPH from two liver donors (HL-#131 and HL-#133), the treatment of compound DL5016, similar to CITCO [2], concentration-dependently induced the expression of CYP2B6 mRNA (Figure 7A). This was not achieved by the amide analogue **1** (figure S1), implying that different gene induction effects can happen even within the same scaffold. Similar to the effect of CITCO, the induction of CYP3A4 by DL5016 was significantly lower compared to CYP2B6. The same trend of results was also observed at the protein level in HPH prepared from the same liver donors (Figure 7B). As expected, the hPXR activator RIF

preferentially induced CYP3A4 over CYP2B6 at both mRNA and protein levels in the same liver donors.

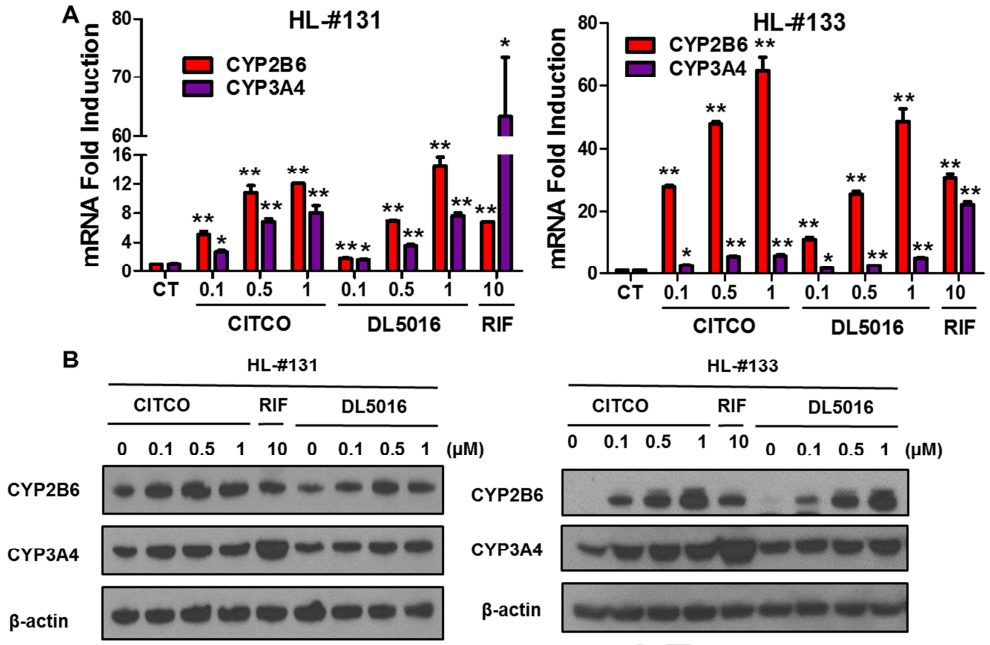


Figure 7. hCAR regulated induction of CYP2B6 and CYP3A4 in HPH. (A) CYP2B6 and CYP3A4 mRNA levels were measured in HPH prepared from human liver donors (HL-#131 and HL-#133). The HPH were treated with CITCO, RIF, DL5016 or the vehicle control (0.1% DMSO). (B) Representative immunoblots of CYP2B6 and CYP3A4 proteins in HPH from liver donors #131 and #133. RT-PCR data obtained from three independent experiments were expressed as mean ± SD normalized against vehicle control. *, $P < 0.05$; **, $P < 0.01$.

2.6. DL5016 causes hCAR nuclear translocation in HPH

It has been well-documented that chemically-stimulated activation causes hCAR to translocate from the cytoplasm to the nucleus in HPH as well as *in vivo* [40]. As shown in Figure 8, compound DL5016 triggered hCAR nuclear translocation using Ad-EYEP-hCAR-

infected HPH treated with vehicle control (0.1% DMSO), CITCO, or DL5016. While in the vehicle control-treated HPH, Ad-EYFP-hCAR mostly remains in the cytoplasm, it is efficiently translocated into the nucleus upon treatment of the positive control CITCO and compound DL5016. These results confirmed that compound DL5016 mediates the first, essential step in hCAR activation.

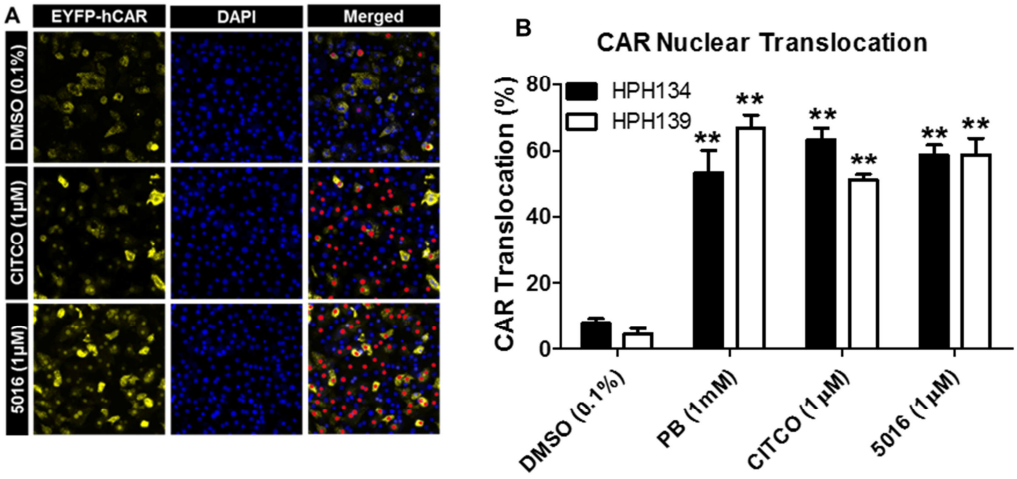


Figure 8. DL5016 promotes translocation of hCAR from cytoplasm to nucleus. HPH were infected with Ad-EYFP-hCAR overnight and then treated with DMSO (0.1%), phenobarbital (PB, 1 mM), CITCO (1 μM), or DL5016 (1 μM) for 8 h. Cells were fixed, stained with DAPI to represent the nucleus, and imaged. In representative images from HPH139, hCAR is sequestered in the cytoplasm of HPH without treatment, while both CITCO and DL5016 promote significant nuclear accumulation of hCAR, denoted with a red mask (A). Quantitation of hCAR translocation reveals DL5016 translocates hCAR similar to agonists PB and CITCO, where CAR translocation (%) is calculated based on the percent of cells that expressing hCAR where over 50% of the nucleus overlaps with EYFP-hCAR (B). **, $P < 0.01$.

2.7. DL5016 promotes CHOP-mediated cytotoxicity of SU-DHL-4 cells in the HPH/SU-DHL-4 co-culture system.

The HPH/SU-DHL-4 cell co-culture model has been proved to be an excellent in vitro compound evaluation assay that mimics the *in vivo* conditions of human body and allows simultaneous studies of hepatic metabolism and extrahepatic anti-cancer effects [2]. To evaluate the anti-cancer effects of DL5016, different concentrations of the CPA-based regimen CHOP were added to the co-cultures with the presence of vehicle (0.1% DMSO) or DL5016 (1 μM) (Figure 9A). The results indicate that co-treatment with DL5016 significantly enhanced CHOP-induced cytotoxicity in SU-DHL-4 cells. Specifically, in the presence of DL5016, the cytotoxicity of 100 μM CHOP was similar or even stronger than that of 500 μM CHOP alone. We also studied the temporal effects of CHOP in the presence of DL5016 (Figure 9B). CHOP caused cytotoxicity in SU-DHL-4 cells time-dependently, and addition of DL5016 dramatically enhanced CHOP-induced cytotoxicity in 24 h after co-treatment. A control experiment demonstrated that compound DL5016, by itself, did not show cytotoxicity in the co-cultured SU-DHL-4 cells.

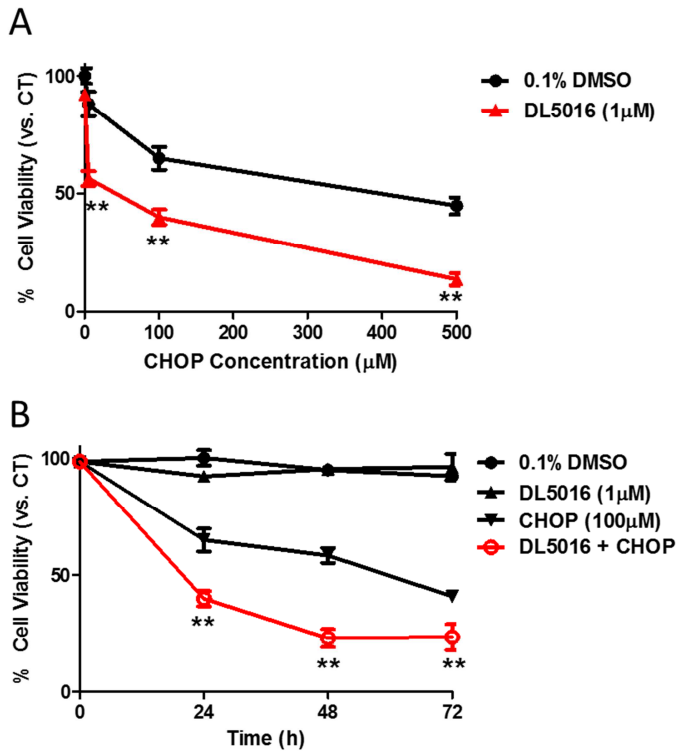


Figure 9. Activation of hCAR enhanced cytotoxicity against SU-DHL-4 cells by CPA-based regimen CHOP in the HPH/SU-DHL-4 co-culture system. (A) Effects of hCAR activation on the concentration-dependent anti-cancer effects of CHOP in SU-DHL-4 cells. The HPH/SU-DHL-4 co-cultures were treated with vehicle control (0.1% DMSO) or CHOP (5, 100 and 500 μM) in the presence and absence of DL5016 (1 μM). Cell viability was analyzed 24 h after the co-treatment. (B) Effects of hCAR activation on the time-dependent cytotoxicity of CHOP in SU-DHL-4 cells. The HPH/SU-DHL-4 co-cultures were treated with vehicle control (0.1% DMSO) or CHOP (100 μM) in the presence and absence of DL5016 (1 μM). Data obtained from three independent measurements were expressed as mean ± SD normalized as the percentage of viability of vehicle control. Statistical significance between the treatment groups CHOP and DL5016/CHOP were analyzed; **, $P < 0.01$.

3. Conclusion

In summary, the development of potent and selective hCAR agonists represents a promising approach to sensitize CPA-based chemotherapies by improving the metabolic efficiency of CPA. As the most well-known hCAR agonist, CITCO is widely utilized in various biological studies. However, CITCO is not stable, which undergoes an isomerization reaction in solution. Through structural modification of CITCO, we have developed a novel compound, DL5016. DL5016 is chemically stable while also potently and selectively activates hCAR over hPXR. DL5016 induces the expression of hCAR target genes on both the mRNA and protein levels. It causes translocation of hCAR from cytoplasm to the nucleus. Addition of DL5016 dramatically decreases the efficacious dose of the CPA-based combination regimen CHOP.

4. Experimental section

4.1. General information

All reagents and solvents were of analytical grade and used without further purification. Reactions were monitored using thin-layer chromatography (TLC) on commercial silica-gel plates (GF254). UV spectra were obtained on a Nanodrop 2000c spectrophotometer. Flash column chromatography was performed on silica gel (200−300 mesh). NMR spectra were obtained on a Varian INOVA 400 MHz NMR spectrometer at 25 °C. Chemical shifts were reported as δ values (parts per million) using the residual solvent peak as an internal reference. Data for ^{1}H NMR were reported in the following order: chemical shift, multiplicity (s, singlet; d, doublet; t, triplet; sept, septuplet; dd, double doublet; dt, double triplet; m, multiplet), coupling constant J (Hz), number of protons. Data for ^{13}C NMR were reported as δ values (parts per million). High-resolution mass spectra (HRMS) were obtained on a JEOL AccuTOF with

ESI/APCI ion sources coupled to an Agilent 1100 HPLC system. HPLC analysis was performed on a Shimadzu HPLC fitted with a C18 reversed-phase column (Phenomenex, luna 5 μM C18(2) 4.6 mm × 100 mm) with a flow rate of 0.8 mL/min using CH_3OH/H_2O 75:25 with 0.1% NH_4OAc or CH_3CN/H_2O 80:20 mobile phase. The purity of final products was > 95%.

General Procedure A: A solution of 2-aminothiazole (5 mmol, 1 equiv) and bromomethyl ketone (5 mmol, 1 equiv) in EtOH (30 mL) was heated under reflux for 16 h. The solvent was removed under reduced pressure, and saturated $NaHCO_3$ (30 mL) was added to the remaining solid. The mixture was extracted using EtOAc (30 mL × 3), and the organic layers were combined, and dried over Na_2SO_4. The concentrated crude product was dried overnight under vacuum to get the crude imidazothiazole that was used directly in the next step. The Vilsmeier reagent was prepared by dropping $POCl_3$ (16.5 mmol, 3.3 equiv) into a solution of DMF (5 mmol, 1.0 equiv) in $CHCl_3$ (5 mL) at 0 °C. To the resulting mixture at 0-5 °C was added a solution of imidazothiazole (5 mmol) in $CHCl_3$ (30 mL) dropwise. The reaction was warmed to the room temperature over 1 h, and then heated under reflux for an additional 5 h. The solvent was removed under reduced pressure and the resulting residue was poured onto ice. The crude aldehyde was collected by filtration and further purified using flash chromatography.

General Procedure B: A solution of oxazol-2-amine (5 mmol, 1 equiv) and bromomethyl ketone (5 mmol, 1 equiv) in THF (30 mL) and CH_3CN (30 mL) was allowed to stir at room temperature for 24 h. The precipitation from the reaction mixture was collected by filtration and then washed using CH_3CN. To a mixture of the resulting solid in toluene (50 mL) at 0 ^oC was added titanium (IV) chloride (0.94 g, 5 mmol) as a solution in toluene (5 mL) over 30 min. The reaction mixture was heated at 100 ^oC for an additional 3 h, and cooled. The solvent was removed by rotary evaporation, and ice was added to the residue. The pH value of the resulting

mixture was adjusted to 9 using Na_2CO_3, and the resulting solution was extracted using EtOAc (30 mL × 3). The organic layers were combined, and dried over Na_2SO_4. The concentrated crude product was dried overnight under vacuum to get the imidazooxazole that was used without further purification. The Vilsmeier reagent was prepared by dropping $POCl_3$ (16.5 mmol, 3.3 equiv) into a solution of DMF (5 mmol, 1.0 equiv) in $CHCl_3$ (5 mL) at 0 °C. To the resulting mixture at 0-5 °C was added a solution of imidazothiazole (5 mmol) in $CHCl_3$ (30 mL) dropwise. The reaction was warmed to the room temperature over 1 h, and then heated under reflux for an additional 5 h. The solvent was removed under reduced pressure and the resulting residue was poured onto ice. The crude aldehyde was collected by filtration and further purified using flash chromatography.

General Procedure C: To a solution of aldehyde (1 mmol) in EtOH (5 mL) was added amine (1 mmol) followed by AcOH (5 mmol, 5 equiv). The reaction mixture was heated under reflux overnight, and then concentrated. To a solution of the resulting residue in MeOH at 0 °C was added $NaBH_4$ (5 mmol, 5 equiv). The reaction mixture was allowed to stir at room temperature overnight. The reaction mixture was partitioned between saturated NH_4Cl (30 mL) and EtOAc (30 mL). The aqueous layer was extracted using EtOAc (30 mL × 2) and the combined organics were washed with brine (45 mL), dried (Na_2SO_4) and concentrated. The crude product was purified by flash chromatography (1-5% MeOH in CH_2Cl_2) to give the desired product.

*4.1.1. 6-(4-Chlorophenyl)-N-(3,4-dichlorophenethyl)imidazo[2,1-b]thiazole-5-carboxamide (**1**)*

Compound **1** was synthesized starting with **33** and 2-(3,4-dichlorophenyl)ethan-1- amine using the first half of General Procedure C (50%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.36 (s, 1H), 8.28 (s, 1H), 8.51 (d, J = 7.2 Hz, 2H), 7.43-7.36 (m, 4H), 7.07 (d, J = 7.6 Hz, 1H), 6.91 (s, 1H),

3.85 (t, *J* = 6.8 Hz, 2H), 2.96 (t, *J* = 6.8 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 152.8, 150.0, 140.3, 134.4, 132.2, 132.1, 131.0, 130.24, 130.17, 129.8, 128.9, 128.6, 121.8, 120.7, 112.8, 62.4, 36.9; HRMS (ESI): Exact mass calcd for $C_{20}H_{15}Cl_3N_3S$ $[M+H]^+$ 434.0052, found 434.0037; HPLC analysis: retention time = 12.1 min, peak area 98.1%, 80:20 CH_3CN/H_2O

*4.1.2. N-((6-(4-Chlorophenyl)imidazo[2,1-b]thiazol-5-yl)methyl)-2-(3,4-dichlorophenyl)ethan-1-amine (**2**)*

Compound **2** was synthesized using the second half of General Procedure C (64%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.57 (d, *J* = 8.4 Hz, 2H), 7.48 (d, *J* = 4.4 Hz, 1H), 7.38 (d, *J* = 8.4 Hz, 2H), 7.32 (d, *J* = 8.0 Hz, 1H), 7.25 (d, *J* = 6.8 Hz, 1H), 6.96 (d, *J* = 8.8 Hz, 1H), 6.75 (d, *J* = 4.8 Hz, 1H), 4.12 (s, 2H), 2.87 (t, *J* = 6.0 Hz, 2H), 2.71 (t, *J* = 6.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.1, 143.7, 140.1, 133.1, 132.3, 130.6, 130.3, 130.2, 129.0, 128.7, 128.1, 120.3, 118.7, 111.9, 49.6, 43.7, 35.3; HRMS (ESI): Exact mass calcd for $C_{20}H_{17}Cl_3N_3S$ $[M+H]^+$ 436.0209, found 436.0207; HPLC analysis: retention time = 18.5 min, peak area 97.4%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.3. N-((6-(4-Chlorophenyl)imidazo[2,1-b]thiazol-5-yl)methyl)-2-(3,4-dichlorophenyl)-N-methylethan-1-amine (**3**)*

To a solution of compound **2** (130 mg, 0.3 mmol) and K_2CO_3 (50 mg, 0.36 mmol) in DMF was added MeI (47 mg, 0.33 mmol). The reaction mixture was stirred at 60 ^{o}C overnight and then cooled to room temperature. The resulting residue was dissolved in H_2O (50 mL) to give a yellow solution. The resulting solution was extracted using CH_2Cl_2 (30 mL × 3), and the combined organic layers were dried over Na_2SO_4. The solvent was removed by rotary evaporation. The crude product was purified by flash chromatography to give compound **3** (83

mg, 61%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.61 (d, J = 8.8 Hz, 2H), 7.37 (d, J = 8.8 Hz, 2H), 7.26 (d, J = 8.0 Hz, 1H), 7.16 (d, J = 1.6 Hz, 1H), 7.08 (d, J = 4.0 Hz, 1H), 6.87 (d, J = 8.8 Hz, 1H), 6.62 (d, J = 4.0 Hz, 1H), 3.79 (s, 2H), 2.69 (t, J = 6.0 Hz, 2H), 2.62 (t, J = 5.6 Hz, 2H), 2.26 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.1, 144.2, 140.7, 133.2, 133.0, 132.1, 130.6, 130.2, 129.9, 129.2, 128.6, 128.0, 119.4, 118.7, 111.6, 57.6, 52.4, 41.2, 32.6; HRMS (ESI): Exact mass calcd for $C_{21}H_{19}Cl_3N_3S$ $[M+H]^+$ 450.0365, found 450.0351; HPLC analysis: retention time = 13.1 min, peak area 99.6%, 80:20 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.4. 6-(4-Chlorophenyl)imidazo[2,1-b]thiazole-5-carboxylic acid (**34**)*

To a solution of intermediate **33** (262 mg, 1 mmol) in the mixture of *t*-butanol (10 mL) and H_2O (10 mL) was added 2-methyl-2butene (2 M in THF, 6.7 mmol), sodium phosphate monobasic (936 mg, 7.8 mmol), and sodium chlorite (1.1 g, 10 mmol). The reaction mixture was stirred at room temperature overnight. EtOAc (50 mL), then H_2O (30 mL) were added. After stirring for an additional 2 h, the mixture was extracted using EtOAc (30 mL × 3). The combined oragnic layers were washed using brine, dried over Na_2SO_4, and concentrated. The crude material was purified by flash chromatography to give carboxylic acid **34** (236 mg, 85%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.23 (d, J = 4.0 Hz, 1H), 7.85 (d, J = 8.4 Hz, 2H), 7.43 (d, J = 8.4 Hz, 2H), 7.05 (d, J = 4.8 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 165.7, 157.3, 156.1, 138.3, 137.8, 136.5, 133.5, 132.9, 127.0, 120.8.

*4.1.5. 6-(4-Chlorophenyl)-N-(3,4-dichlorophenethyl)imidazo[2,1-b]thiazole-5-carboxamide (**4**)*

To a solution of carboxylic acid **34** (139 mg, 0.5 mmol) in DMF (10 mL) was added EDCI (120 mg, 0.63 mmol) and DMAP (77 mg, 0.63 mmol), followed by 2-(3,4-dichlorophenyl)ethan-1-amine (95 mg, 0.5 mmol). Upon completion of the reaction (followed by TLC), the reaction

mixture was partitioned between $NaHCO_3$ (10 mL) and EtOAc (25 mL). The aqueous layer was extracted by EtOAc (25 mL × 2). The combined organic layers were dried over Na_2SO_4, and concentrated. The crude material was purified by flash chromatography to give compound **4** (146 mg, 65%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.36 (d, J = 4.4 Hz, 1H), 7.44 (d, J = 7.6 Hz, 2H), 7.36-7.34 (m, 3H), 7.19 (s, 1H), 6.98 (d, J = 4.4 Hz, 1H), 6.91 (d, J = 8.0 Hz, 1H), 5.70 (br s, 1H), 3.57 (q, J = 6.0 Hz, 2H), 2.76 (t, J = 6.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.8, 152.0, 147.4, 138.6, 135.5, 132.6, 130.7, 130.6, 130.5, 129.2, 127.9, 121.6, 117.5, 113.5, 39.9, 34.5; HRMS (ESI): Exact mass calcd for $C_{20}H_{15}Cl_3N_3OS$ $[M+H]^+$ 450.0001, found 450.0006; HPLC analysis: retention time = 4.2 min, peak area 96.2%, 80:20 CH_3CN/H_2O.

*4.1.6. (6-(4-Chlorophenyl)imidazo[2,1-b]thiazol-5-yl)methanol (**35**)*

To a solution of compound **33** (5 mmol) in MeOH (200 mL) at 0 ^{o}C was added $NaBH_4$ (15 mmol, 3 equiv). The resulting mixture was warmed to room temperature and stirred for 1 h, and concentrated. To the resulting residue was partitioned between saturated Na_2CO_3 (50 mL) and EtOAc (50 mL). The aqueous layer was extracted twice using EtOAc (50 mL × 2). The solvent was removed and the crude material was purified using flash chromatography to give alcohol **35** (75%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.95 (d, J = 8.8 Hz, 1H), 7.78 (d, J = 8.8 Hz, 2H), 7.50 (d, J = 8.0 Hz, 2H), 5.51 (br s, 1H), 4.80 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 153.2, 147.4, 138.6, 136.9, 133.9, 133.7, 128.5, 124.3, 118.4, 58.1.

*4.1.7. 6-(4-Chlorophenyl)-5-((3,4-dichlorophenethoxy)methyl)imidazo[2,1-b]thiazole **(5)***

To a solution of alcohol **35** (0.5 mmol) in CH_2Cl_2 (10 mL) at 0 ^{o}C was added TEA (0.55 mmol, 1.1 equiv) and methanesulfonyl chloride (0.55 mmol, 1.1 equiv). The mixture was stirred at 0 ^{o}C for an additional 2 h, and concentrated to provide crude compound **36**. To a solution of 2-

(3,4-dichlorophenyl)ethan-1-ol (0.5 mmol) and K_2CO_3 (1 mmol, 2 equiv) in DMF was added compound **36** (0.5 mmol). The reaction mixture was stirred at 60 ^oC overnight, cooled, and partitioned between H_2O (50 mL) and EtOAc (30 mL). The aqueous layer was extracted twice using EtOAc (30 mL × 2). The combined organic layers were washed using brine, dried over Na_2SO_4, and concentrated. The crude material was purified by flash chromatography to give compound **5** (28%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.78 (d, J = 8.0 Hz, 2H), 7.60 (d, J = 8.0 Hz, 2H), 7.53 (d, J = 8.0 Hz, 1H), 7.48 (d, J = 3.6 Hz, 2H), 7.38 (d, J = 3.6 Hz, 1H), 7.20 (d, J = 8.4 Hz, 1H), 7.01 (d, J = 3.6 Hz, 1H), 4.97 (s, 2H), 3.88 (t, J = 5.6 Hz, 2H), 3.04 (t, J = 5.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.6, 145.0, 139.3, 133.6, 132.5, 132.2, 130.8, 130.3, 130.2, 129.0, 128.8, 128.3, 118.7, 117.8, 112.7, 70.0, 62.7, 35.3; HRMS (ESI): Exact mass calcd for $C_{20}H_{16}Cl_3N_2OS$ $[M+H]^+$ 437.0049, found 437.0051; HPLC analysis: retention time = 5.7 min, peak area 97.6%, 80:20 CH_3CN/H_2O.

*4.1.8. 6-(4-Chlorophenyl)-2-methylimidazo[2,1-b]thiazole-5-carbaldehyde (**40**)*

Compound **40** was synthesized using **37** as a starting material following General Procedure A (67%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.84 (s, 1H), 8.11 (s, 1H), 7.72 (d, J = 8.8 Hz, 2H), 7.48 (d, J = 7.6 Hz, 2H), 2.52 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.6, 155.7, 135.7, 131.1, 130.2, 129.3, 129.2, 123.9, 118.0, 14.0.

*4.1.9. 6-(4-Chlorophenyl)imidazo[2,1-b]oxazole-5-carbaldehyde (**41**)*

Compound **41** was synthesized using **38** as a starting material following General Procedure B (65%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.85 (s, 1H), 7.97 (s, 1H), 7.74 (d, J = 8.8 Hz, 2H), 7.57 (s, 1H), 7.47 (d, J = 8.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.9, 154.7, 139.2 (2C), 136.0, 131.0, 130.1 (2C), 129.2 (2C), 120.4, 113.9.

*4.1.10. 2-(4-Chlorophenyl)imidazo[1,2-a]pyrimidine-3-carbaldehyde (**42**)*

Compound **42** was synthesized using **39** as a starting material following General Procedure A (87%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.1 (s, 1H), 9.87 (d, J = 5.2 Hz, 1H), 8.84 (d, J = 1.6 Hz, 1H), 7.85 (d, J = 8.4 Hz, 2H), 7.52 (d, J = 7.6 Hz, 2H), 7.22-7.19 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.7, 157.8, 154.7, 150.3, 136.8, 136.4, 131.1, 130.2, 129.3, 118.9, 111.5.

*4.1.11. N-((6-(4-Chlorophenyl)-2-methylimidazo[2,1-b]thiazol-5-yl)methyl)-2-(3,4-dichlorophenyl)ethan-1-amine **(6)***

Compound **6** was synthesized using **40** and 2-(3,4-dichlorophenyl)ethan-1-amine as starting material following General Procedure C (22%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.55 (d, J = 7.6 Hz, 2H), 7.38-7.32 (m, 3H), 7.26 (s, 1H), 7.10 (s, 1H), 6.96 (d, J = 7.6 Hz, 1H), 4.06 (s, 2H), 2.88 (t, J = 6.4 Hz, 2H), 2.71 (t, J = 6.8 Hz, 2H), 2.39 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 148.6, 142.4, 140.3, 133.3, 132.9, 132.2, 130.6, 130.1, 128.8, 128.7, 128.1, 126.1, 120.1, 115.0, 49.5, 43.7, 35.3, 14.1; HRMS (ESI): Exact mass calcd for $C_{21}H_{19}Cl_3N_3S$ $[M+H]^+$ 450.0365, found 450.0363; HPLC analysis: retention time = 25.1 min, peak area 99.2%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.12. N-((6-(4-Chlorophenyl)imidazo[2,1-b]oxazol-5-yl)methyl)-2-(3,4-dichlorophenyl)ethan-1-amine (**7**)*

Compound **7** was synthesized using **41** and 2-(3,4-dichlorophenyl)ethan-1-amine as starting material following General Procedure C (57%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.53 (d, J = 7.6 Hz, 2H), 7.35-7.24 (m, 6H), 6.96 (d, J = 8.0 Hz, 1H), 4.03 (s, 2H), 2.84 (t, J = 7.2 Hz, 2H), 2.69 (t, J = 6.8 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 154.8, 140.2, 139.5, 137.1, 133.3, 132.8, 132.2, 130.6, 130.3, 130.1, 128.8, 128.6, 128.2, 116.1, 111.8, 49.7, 43.9, 35.4; HRMS (ESI):

Exact mass calcd for $C_{20}H_{17}Cl_3N_3O$ $[M+H]^+$ 420.0437, found 420.0445; HPLC analysis: retention time = 14.0 min, peak area 98.6%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.13. N-((2-(4-Chlorophenyl)imidazo[1,2-a]pyrimidin-3-yl)methyl)-2-(3,4-dichlorophenyl)ethan-1-amine (**8**)*

Compound **8** was synthesized using **42** and 2-(3,4-dichlorophenyl)ethan-1-amine as starting material following General Procedure C (48%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.54-8.51 (m, 2H), 7.70 (d, J = 8.8 Hz, 2H), 7.44-7.41 (m, 2H), 7.31-7.24 (m, 2H), 6.93 (d, J = 7.6 Hz, 1H), 6.78 (d, J = 4.4 Hz, 1H), 4.24 (s, 2H), 2.89 (t, J = 6.4 Hz, 2H), 2.71 (t, J = 6.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 150.0, 148.1, 144.2, 140.1, 134.2, 132.9, 132.3, 132.2, 130.7, 130.3, 130.2, 130.0, 128.8, 128.1, 116.4, 108.1, 49.6, 43.0, 35.3; HRMS (ESI): Exact mass calcd for $C_{21}H_{18}Cl_3N_4$ $[M+H]^+$ 431.0597, found 431.0588; HPLC analysis: retention time = 11.5 min, peak area 96.5%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.14. 2-(4-Chlorophenyl)-N-((6-(4-chlorophenyl)imidazo[2,1-b]thiazol-5-yl)methyl)ethan-1-amine (**9**)*

Compound **9** was synthesized using **33** and 2-(4-chlorophenyl)ethan-1-amine as starting material following General Procedure C (28%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.56 (d, J = 8.8 Hz, 2H), 7.50 (d, J = 4.0 Hz, 1H), 7.38 (d, J = 8.8 Hz, 2H), 7.24 (d, J = 8.8 Hz, 2H), 7.06 (d, J = 7.6 Hz, 2H), 6.75 (d, J = 4.8 Hz, 1H), 4.12 (s, 2H), 2.88 (t, J = 7.2 Hz, 2H), 2.74 (t, J = 7.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.1, 143.7, 138.1, 133.1, 132.0, 130.0, 129.0, 128.7, 128.6, 126.5, 120.3, 118.8, 111.9, 49.9, 43.6, 35.4; HRMS (ESI): Exact mass calcd for $C_{20}H_{18}Cl_2N_3S$ $[M+H]^+$ 402.0598, found 402.0598; HPLC analysis: retention time = 12.7 min, peak area 97.7%, 75:25 CH_3CN/H_2O with 0.1% NH_4OAc.

*4.1.15. 2-(3-Chlorophenyl)-N-((6-(4-chlorophenyl)imidazo[2,1-b]thiazol-5-yl)methyl)ethan-1-amine (**10**)*

Compound **10** was synthesized uisng **33** and 2-(3-chlorophenyl)ethan-1-amine as starting material following General Procedure C (43%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.57-7.53 (m, 3H), 7.38 (d, *J* = 8.4 Hz, 2H), 7.20 (d, *J* = 8.4 Hz, 2H), 7.16 (s, 1H), 7.02 (d, *J* = 5.6 Hz, 1H), 6.76 (d, *J* = 4.0 Hz, 1H), 4.12 (s, 2H), 2.90 (t, *J* = 7.2 Hz, 2H), 2.75 (t, *J* = 6.8 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.1, 143.7, 141.8, 134.2, 133.1, 129.7, 129.0, 128.8, 128.7, 126.9, 126.5, 120.2, 118.8, 111.9, 49.8, 43.7, 35.7; HRMS (ESI): Exact mass calcd for $C_{20}H_{18}Cl_2N_3S$ $[M+H]^+$ 402.0598, found 402.0599; HPLC analysis: retention time = 12.5 min, peak area 95.7%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.16. N-((6-(4-Chlorophenyl)imidazo[2,1-b]thiazol-5-yl)methyl)-2-(4-fluorophenyl)ethan-1-amine (**11**)*

Compound **11** was synthesized using **33** and 2-(4-fluorophenyl)ethan-1-amine as starting material following General Procedure C (44%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.57 (d, *J* = 8.4 Hz, 2H), 7.50 (d, *J* = 4.8 Hz, 1H), 7.37 (d, *J* = 8.8 Hz, 2H), 7.11-7.08 (m, 2H), 6.96 (t, *J* = 8.8 Hz, 2H), 6.75 (d, *J* = 4.8 Hz, 1H), 4.11 (s, 2H), 2.88 (t, *J* = 6.8 Hz, 2H), 2.74 (t, *J* = 6.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 161.5 (*J* = 244.1 Hz,), 149.0, 143.5, 135.4, 133.2, 133.0, 130.0 (*J* = 7.4 Hz), 129.0, 128.6, 120.6, 118.8, 115.2 (*J* = 22.4 Hz), 111.8, 50.2, 43.8, 35.3; HRMS (ESI): Exact mass calcd for $C_{20}H_{18}ClFN_3S$ $[M+H]^+$ 386.0894, found 386.0894; HPLC analysis: retention time = 8.2 min, peak area 95.3%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.17. 2-(4-Bromophenyl)-N-((6-(4-chlorophenyl)imidazo[2,1-b]thiazol-5-yl)methyl)ethan-1-amine (**12**)*

Compound **12** was synthesized using **33** and 2-(4-bromophenyl)ethan-1-amine as starting material following General Procedure C (38%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.55 (d, J = 8.8 Hz, 2H), 7.46 (d, J = 4.8 Hz, 1H), 7.39-7.36 (m, 4H), 7.01 (d, J = 8.0 Hz, 2H), 6.74 (d, J = 8.4 Hz, 1H), 4.09 (s, 2H), 2.87 (t, J = 6.8 Hz, 2H), 2.71 (t, J = 6.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.0, 143.6, 138.8, 133.1, 131.5, 130.4, 129.0, 128.8, 128.6, 120.5, 120.0, 118.8, 111.9, 49.9, 43.7, 35.6; HRMS (ESI): Exact mass calcd for $C_{20}H_{18}BrClN_3S$ $[M+H]^+$ 446.0093, found 446.0101; HPLC analysis: retention time = 14.1 min, peak area 95.4%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.18. 4-(2-(((6-(4-Chlorophenyl)imidazo[2,1-b]thiazol-5-yl)methyl)amino)ethyl)phenol (**13**)*

Compound **13** was synthesized using **33** and 4-(2-aminoethyl)phenol as starting material following General Procedure C (31%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.54-7.49 (m, 3H), 7.36 (d, J = 8.8 Hz, 2H), 6.97 (d, J = 8.4 Hz, 2H), 6.72 (d, J = 7.2 Hz, 2H), 4.09 (s, 2H), 2.87 (t, J = 6.0 Hz, 2H), 2.71 (t, J = 6.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 154.5, 149.0, 143.4, 133.1, 132.9, 131.3, 129.7, 129.0, 128.7, 120.7, 118.8, 115.5, 112.0, 50.3, 43.7, 35.0; HRMS (ESI): Exact mass calcd for $C_{20}H_{19}ClN_3OS$ $[M+H]^+$ 384.0937, found 384.0931; HPLC analysis: retention time = 3.5 min, peak area 97.3%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.19. N-((6-(4-Chlorophenyl)imidazo[2,1-b]thiazol-5-yl)methyl)-2-phenylethan-1-amine (**14**)*

Compound **14** was synthesized using **33** and 2-phenylethan-1-amine as starting material following General Procedure C (42%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.56 (d, J = 8.8 Hz, 2H), 7.51 (d, J = 8.0 Hz, 1H), 7.37 (d, J = 8.8 Hz, 2H), 7.29 (t, J = 8.0 Hz, 2H), 7.23 (d, J = 8.0 Hz, 1H), 7.16 (d, J = 7.2 Hz, 2H), 6.73 (d, J = 4.0 Hz, 1H), 4.09 (s, 2H), 2.92 (t, J = 6.4 Hz, 2H), 2.78 (t, J = 6.0 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.0, 143.5, 139.7, 133.2, 133.0,

129.0, 128.7, 128.6, 128.5, 126.3, 120.7, 118.9, 111.8, 50.2, 43.8, 36.1; HRMS (ESI): Exact mass calcd for $C_{20}H_{19}ClN_3S$ $[M+H]^+$ 368.0988, found 368.0989; HPLC analysis: retention time = 8.6 min, peak area 97.5%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.20. N-Benzyl-1-(6-(4-chlorophenyl)imidazo[2,1-b]thiazol-5-yl)methanamine (**15**)*

Compound **15** was synthesized using **33** and phenylmethanamine as starting material following General Procedure C (38%): ^1H NMR (400 MHz, $CDCl_3$): δ 7.60 (d, J = 4.8 Hz, 1H), 7.55 (d, J = 8.8 Hz, 2H), 7.35-7.25 (m, 7H), 6.79 (d, J = 3.6 Hz, 1H), 4.10 (s, 2H), 3.79 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.0, 143.7, 139.6, 133.1, 133.0, 128.9, 128.6, 128.5, 128.2, 127.2, 120.6, 118.7, 112.0, 53.1, 42.8; HRMS (ESI): Exact mass calcd for $C_{19}H_{17}ClN_3S$ $[M+H]^+$ 354.0832, found 354.0834; HPLC analysis: retention time = 7.9 min, peak area 95.9%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.21. N-((6-(4-Chlorophenyl)imidazo[2,1-b]thiazol-5-yl)methyl)-2-(1H-indol-3-yl)ethan-1-amine (**16**)*

Compound **16** was synthesized using **33** and 2-(1*H*-indol-3-yl)ethan-1-amine as starting material following General Procedure C (49%): ^1H NMR (400 MHz, $CDCl_3$): δ 8.27 (br s, 1H), 7.59 (d, J = 8.0 Hz, 1H), 7.53-7.49 (m, 3H), 7.35 (d, J = 8.0 Hz, 1H), 7.28-7.20 (m, 3H), 7.12 (t, J = 7.6 Hz, 1H), 6.94 (s, 1H), 6.70 (d, J = 4.8 Hz, 1H), 4.08 (s, 2H), 3.01-2.95 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 148.9, 143.4, 136.4, 133.1, 132.9, 128.9(2C), 128.6, 127.3, 122.1, 119.3, 118.8, 118.7, 113.4, 111.9, 111.3, 49.1, 43.8, 25.5; HRMS (ESI): Exact mass calcd for $C_{22}H_{20}ClN_4S$ $[M+H]^+$ 407.1097, found 407.1096; HPLC analysis: retention time = 6.6 min, peak area 95.7%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.22. N-((6-(4-Chlorophenyl)imidazo[2,1-b]thiazol-5-yl)methyl)-2,3-dihydro-1H-inden-2-amine (**17**)*

Compound **17** was synthesized using **33** and 2,3-dihydro-1*H*-inden-2-amine as starting material following General Procedure C (26%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.66 (d, J = 4.4 Hz, 1H), 7.62 (d, J = 8.4 Hz, 2H), 7.40 (d, J = 8.0 Hz, 2H), 7.21-7.15 (m, 4H), 6.78 (d, J = 4.8 Hz, 1H), 4.19 (s, 2H), 3.69-3.62 (m, 1H), 3.15 (dd, J = 7.2 Hz, J = 8.4 Hz, 2H), 2.89 (dd, J = 5.6 Hz, J = 10.0 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.0, 143.4, 141.4, 133.2, 133.1, 129.0, 128.7, 126.5, 124.7, 120.7, 118.9, 112.0, 59.1, 42.3, 39.9; HRMS (ESI): Exact mass calcd for $C_{21}H_{19}ClN_3S$ $[M+H]^+$ 380.0988, found 380.0991; HPLC analysis: retention time = 11.3 min, peak area 95.3%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.23. N-((6-(4-Chlorophenyl)imidazo[2,1-b]thiazol-5-yl)methyl)naphthalen-2-amine (**18**)*

Compound **18** was synthesized using **33** and naphthalen-2-amine as starting material following General Procedure C (15%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.73-7.66 (m, 5H), 7.62 (d, J = 7.6 Hz, 1H), 7.48 (d, J = 4.4 Hz, 1H), 7.43-7.28 (m, 3H), 7.26 (t, J = 7.2 Hz, 1H), 6.93 (d, J = 7.6 Hz, 2H), 6.84 (d, J = 4.8 Hz, 1H), 4.71 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.5, 145.0, 144.4, 134.9, 133.5, 132.6, 129.3, 128.9, 128.8, 127.9, 127.7, 126.7, 126.1, 122.7, 118.8, 117.8, 112.9, 105.0, 39.2; HRMS (ESI): Exact mass calcd for $C_{22}H_{17}ClN_3S$ $[M+H]^+$ 390.0832, found 390.0839; HPLC analysis: retention time = 14.0 min, peak area 95.9%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.24. 6-Ethylimidazo[2,1-b]thiazole-5-carbaldehyde (**45a**)*

Compound **45a** was synthesized using 1-bromobutan-2-one **43a** and thiazol-2-amine as starting material following General Procedure A (56%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.81 (s,

1H), 8.26 (d, *J* = 3.6 Hz, 1H), 6.98 (d, *J* = 4.8 Hz, 1H), 2.99 (q, *J* = 8.0 Hz, 2H), 1.41 (t, *J* = 8.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): *δ* 175.6, 162.8, 155.6, 123.8, 121.2, 114.0, 21.7, 14.4.

*4.1.25. 6-(3-Chlorophenyl)imidazo[2,1-b]thiazole-5-carbaldehyde (**45b**)*

Compound **45b** was synthesized using 2-bromo-1-(3-chlorophenyl)ethan-1-one **43b** and thiazol-2-amine as starting material following General Procedure A (63%): ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 9.89 (s, 1H), 8.37 (d, *J* = 4.8 Hz, 1H), 7.79 (s, 1H), 7.65 (d, *J* = 6.4 Hz, 1H), 7.43-7.39 (m, 2H), 7.07 (d, *J* = 4.0 Hz, 1H), ^{13}C NMR (100 MHz, $CDCl_3$): *δ* 177.7, 156.2, 155.6, 135.0, 134.1, 130.2, 129.7, 129.0, 127.2, 124.1, 121.5, 115.1.

*4.1.26. 6-(p-Tolyl)imidazo[2,1-b]thiazole-5-carbaldehyde (**45c**)*

Compound **45c** was synthesized using 2-bromo-1-(p-tolyl)ethan-1-one **43c** and thiazol-2-amine as starting material following General Procedure A (65%): ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 9.89 (s, 1H), 8.37 (d, *J* = 4.8 Hz, 1H), 7.69 (d, *J* = 8.0 Hz,2H), 7.31 (d, *J* = 8.0 Hz, 2H), 7.03 (d, *J* = 4.8 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): *δ* 178.1, 158.4, 155.6, 139.9, 129.7, 129.0, 123.9, 121.5, 114.4, 21.4.

*4.1.27. 6-(4-(Trifluoromethyl)phenyl)imidazo[2,1-b]thiazole-5-carbaldehyde (**45d**)*

Compound **45d** was synthesized using 2-bromo-1-(4-(trifluoromethyl)phenyl) ethan-1-one **43d** and thiazol-2-amine as starting material following General Procedure A (55%): ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 9.93 (s, 1H), 8.41 (d, *J* = 4.0 Hz, 1H), 7.93 (d, *J* = 8.0 Hz, 2H), 7.78 (d, *J* = 8.0 Hz, 2H), 7.11 (d, *J* = 4.4 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): *δ* 177.5, 155.9, 155.6, 135.9, 131.1 (*J* = 32.8 Hz), 129.3, 125.8 (*J* = 3.4 Hz), 124.3, 123.9 (*J* = 270.9 Hz), 121.4, 115.3.

*4.1.28. 6-(4-Fluorophenyl)imidazo[2,1-b]thiazole-5-carbaldehyde (**45e**)*

Compound **45e** was synthesized using 2-bromo-1-(4-fluorophenyl)ethan-1-one **43e** and thiazol-2-amine as starting material following General Procedure A (58%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.86 (s, 1H), 8.38 (d, J = 4.8 Hz, 1H), 7.78 (t, J = 8.0 Hz, 2H), 7.19 (t, J = 7.6 Hz, 2H), 7.07 (d, J = 4.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.7, 163.8 (J = 248.5 Hz), 156.6, 155.4, 130.9 (J = 7.4 Hz), 128.3, 123.8, 121.5, 116.2 (J = 20.8 Hz), 115.0.

*4.1.29. 6-Phenylimidazo[2,1-b]thiazole-5-carbaldehyde (**45f**)*

Compound **45f** was synthesized using 2-bromo-1-phenylethan-1-one **43f** and thiazol-2-amine as starting material following General Procedure A (67%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.90 (s, 1H), 8.39 (d, J = 4.0 Hz, 1H), 7.79 (d, J = 6.8 Hz, 2H), 7.50 (d, J = 7.2 Hz, 3H), 7.05 (d, J = 4.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.1, 158.2, 155.6, 132.5, 129.7, 129.1, 128.9, 124.0, 121.5, 114.6.

*4.1.30. 6-(4-Bromophenyl)imidazo[2,1-b]thiazole-5-carbaldehyde (**45g**)*

Compound **45g** was synthesized using 2-bromo-1-(4-bromophenyl)ethan-1-one **43g** and thiazol-2-amine as starting material following General Procedure A (69%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.87 (s, 1H), 8.37 (d, J = 4.8 Hz, 1H), 7.67-7.61 (m, 4H), 7.07 (d, J = 3.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.6, 156.7, 155.7, 132.1, 131.4, 130.5, 124.3, 124.0, 121.5, 114.9.

*4.1.31. 6-(4-Methoxyphenyl)imidazo[2,1-b]thiazole-5-carbaldehyde (**45h**)*

Compound **45h** was synthesized using 2-bromo-1-(4-methoxyphenyl)ethan-1-one **43h** and thiazol-2-amine as starting material following General Procedure A (51%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.88 (s, 1H), 8.37 (d, J = 4.8 Hz, 1H), 7.75 (d, J = 8.8 Hz, 2H), 7.04-7.02 (m, 3H),

3.88 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.0, 161.0, 158.0, 155.5, 130.4, 124.8, 123.6, 121.6, 114.4, 114.3, 55.4.

*4.1.32. 4-(5-Formylimidazo[2,1-b]thiazol-6-yl)benzonitrile (**45i**)*

Compound **45i** was synthesized using 4-(2-bromoacetyl)benzonitrile **43i** and thiazol-2-amine as starting material following General Procedure A (59%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.94 (s, 1H), 8.41 (d, J = 4.0 Hz, 1H), 7.93 (d, J = 8.0 Hz, 2H), 7.81 (d, J = 7.6 Hz, 2H), 7.13 (d, J = 4.8 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.3, 155.8, 155.3, 136.8, 132.7, 129.5, 124.4, 121.5, 118.3, 115.6, 113.2.

*4.1.33. 6-(4-(tert-Butyl)phenyl)imidazo[2,1-b]thiazole-5-carbaldehyde (**45j**)*

Compound **45j** was synthesized using 2-bromo-1-(4-(*tert*-butyl)phenyl)ethan-1-one **43j** and thiazol-2-amine as starting material following General Procedure A (58%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.92 (s, 1H), 8.40 (d, J = 4.4 Hz, 1H), 7.74 (d, J = 8.0 Hz, 2H), 7.54 (d, J = 8.8 Hz, 2H), 7.07 (d, J = 4.0 Hz, 1H), 1.37 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.2, 158.3, 155.6, 153.1, 129.5, 128.8, 125.9, 123.9, 121.5, 114.5, 34.8, 31.2.

*4.1.34. 6-(Naphthalen-2-yl)imidazo[2,1-b]thiazole-5-carbaldehyde (**45k**)*

Compound **45k** was synthesized using 2-bromo-1-(naphthalen-2-yl)ethan-1-one **43k** and thiazol-2-amine as starting material following General Procedure A (69%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.00 (s, 1H), 8.41 (d, J = 4.8 Hz, 1H), 8.27 (s, 1H), 7.99-7.89 (m, 4H), 7.56-7.54 (m, 2H), 7.07 (d, J = 4.4 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.2, 158.1, 155.7, 133.7, 133.2, 129.7, 128.9, 128.8, 128.5, 127.8, 127.1, 126.8, 126.1, 124.2, 121.6, 114.8.

*4.1.35. 2-(3,4-Dichlorophenyl)-N-((6-ethylimidazo[2,1-b]thiazol-5-yl)methyl)ethan-1-amine (**19**)*

Compound **19** was synthesized using **45a** and 2-(3,4-dichlorophenyl)ethan-1-amine as starting material following General Procedure C (42%): ^{1}H NMR (400 MHz, $CDCl_3$): δ *7.35* (d, *J* = 8.8 Hz, 1H), 7.32 (d, *J* = 8.0 Hz, 1H), 7.26 (s, 1H), 6.97 (d, *J* = 8.4 Hz, 1H), 6.67 (d, *J* = 4.8 Hz, 1H), 3.91 (s, 2H), 2.85 (t, *J* = 7.2 Hz, 2H), 2.71 (t, *J* = 7.2 Hz, 2H), 2.64 (q, *J* = 7.2 Hz, 2H), 1.26 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 148.2, 147.2, 140.3, 132.2, 130.6, 130.3, 130.1, 128.1, 118.9, 118.3, 110.8, 49.4, 42.8, 35.4, 21.1, 14.8. HRMS (ESI): Exact mass calcd for $C_{16}H_{18}Cl_2N_3S$ $[M+H]^+$ 354.0598, found 354.0597. HPLC analysis: retention time = 6.1 min, peak area 95.1%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.35. N-((6-(3-Chlorophenyl)imidazo[2,1-b]thiazol-5-yl)methyl)-2-(3,4-dichlorophenyl)ethan-1-amine (**20**)*

Compound **20** was synthesized uisng **45b** and 2-(3,4-dichlorophenyl)ethan-1-amine as starting material following General Procedure C (41%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.64 (s, 1H), 7.49 (t, *J* = 6.8 Hz, 2H), 7.35-7.24 (m, 4H), 6.95 (d, *J* = 8.0 Hz, 2H), 6.76 (d, *J* = 4.8 Hz, 1H); 4.13 (s, 2H), 2.87 (t, *J* = 6.8 Hz, 2H), 2.71 (t, *J* = 6.8 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.2, 143.4, 140.0, 136.3, 134.4, 132.3, 130.6, 130.3, 130.2, 129.7, 128.1, 127.8, 127.3, 125.8, 120.4, 118.8, 112.1, 49.5, 43.6, 35.2; HRMS (ESI): Exact mass calcd for $C_{20}H_{17}Cl_3N_3S$ $[M+H]^+$ 436.0209, found 436.0212; HPLC analysis: retention time = 18.0 min, peak area 97.0%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.36. 2-(3,4-Dichlorophenyl)-N-((6-(p-tolyl)imidazo[2,1-b]thiazol-5-yl)methyl)ethan-1-amine (**21**)*

Compound **21** was synthesized using **45c** and 2-(3,4-dichlorophenyl)ethan-1-amine as starting material following General Procedure C (55%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.51 (d,

J = 8.0 Hz, 2H), 7.46 (d, *J* = 4.8 Hz, 1H), 7.31 (d, *J* = 8.8 Hz, 1H), 7.22 (d, *J* = 7.2 Hz, 3H), 6.94 (d, *J* = 8.0 Hz, 1H), 6.72 (d, *J* = 4.8 Hz, 1H), 4.12 (s, 2H), 2.86 (t, *J* = 6.0 Hz, 2H), 2.69 (t, *J* = 6.4 Hz, 2H), 2.34 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 148.8, 144.8, 140.3, 137.0, 132.2, 131.7, 130.6, 130.3, 130.1, 129.2, 128.1, 127.7, 119.9, 118.7, 111.5, 49.6, 43.7, 35.3, 21.2; HRMS (ESI): Exact mass calcd for $C_{21}H_{20}Cl_2N_3S$ $[M+H]^+$ 416.0755, found 416.0746; HPLC analysis: retention time = 15.0 min, peak area 97.5%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.37. 2-(3,4-Dichlorophenyl)-N-((6-(4-(trifluoromethyl)phenyl)imidazo[2,1-b]thiazol-5-yl)methyl)ethan-1-amine (**22**)*

Compound **22** was synthesized using **45d** and 2-(3,4-dichlorophenyl)ethan-1-amine as starting material following General Procedure C (38%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.75 (d, *J* = 8.8 Hz, 2H), 7.65 (d, *J* = 8.8 Hz, 2H), 7.51 (d, *J* = 4.8 Hz, 1H), 7.31 (d, *J* = 8.0 Hz, 1H), 7.25 (s, 1H), 6.96 (d, *J* = 8.8 Hz, 1H), 6.77 (d, *J* = 4.8 Hz, 1H), 4.15 (s, 2H), 2.89 (t, *J* = 7.2 Hz, 2H), 2.72 (t, *J* = 7.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.4, 143.4, 140.2, 138.1, 132.3, 130.6, 130.3, 130.2, 128.1, 127.8, 126.1, 125.4, 122.9, 120.9, 118.8, 112.3, 49.6, 43.7, 35.2; HRMS (ESI): Exact mass calcd for $C_{21}H_{17}Cl_2F_3N_3S$ $[M+H]^+$ 470.0472, found 470.0459; HPLC analysis: retention time = 20.1 min, peak area 99.2%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.38. 2-(3,4-Dichlorophenyl)-N-((6-(4-fluorophenyl)imidazo[2,1-b]thiazol-5-yl)methyl)ethan-1-amine (**23**)*

Compound **23** was synthesized uisng **45e** and 2-(3,4-dichlorophenyl)ethan-1-amine as starting material following General Procedure C (34%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.56-7.53 (m, 2H), 7.46 (d, *J* = 4.8 Hz, 1H), 7.28 (d, *J* = 8.0 Hz, 1H), 7.07 (t, *J* = 8.4 Hz, 2H), 6.92 (d, *J* = 7.6 Hz, 1H), 6.72 (d, *J* = 4.8 Hz, 1H), 4.07 (s, 2H), 2.83 (t, *J* = 6.0 Hz, 2H), 2.67 (t, *J* = 6.4

Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.4 (J = 245.5 Hz), 148.9, 143.8, 140.1, 132.2, 130.6, 130.3, 130.1, 129.4 (J = 7.5 Hz), 128.1, 119.8, 118.7, 115.4 (J = 22.3 Hz), 111.9, 49.5, 43.5, 35.2; HRMS (ESI): Exact mass calcd for $C_{20}H_{17}Cl_2FN_3S$ $[M+H]^+$ 420.0504, found 420.0515; HPLC analysis: retention time = 11.6 min, peak area 98.8%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.39. 2-(3,4-Dichlorophenyl)-N-((6-phenylimidazo[2,1-b]thiazol-5-yl)methyl)ethan-1-amine (**24**)*

Compound **24** was synthesized using **45f** and 2-(3,4-dichlorophenyl)ethan-1-amine as starting material following General Procedure C (42%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.63 (d, J = 8.0 Hz, 2H), 7.45-7.40 (m, 3H), 7.32 (t, J = 7.6 Hz, 2H), 7.24 (s, 1H), 6.95 (d, J = 8.0 Hz, 1H), 6.74 (d, J = 4.4 Hz, 1H), 4.15 (s, 2H), 2.88 (t, J = 6.4 Hz, 2H), 2.70 (t, J = 6.0 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 148.9, 144.8, 140.3, 134.6, 132.2, 130.6, 130.3, 130.1, 128.5, 128.1, 127.8, 127.3, 120.2, 118.7, 111.7, 49.6, 43.7, 35.4; HRMS (ESI): Exact mass calcd for $C_{20}H_{18}Cl_2N_3S$ $[M+H]^+$ 402.0598, found 402.0594; HPLC analysis: retention time = 10.8 min, peak area 97.0%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.40. N-((6-(4-Bromophenyl)imidazo[2,1-b]thiazol-5-yl)methyl)-2-(3,4-dichlorophenyl)ethan-1-amine (**25**)*

Compound **25** was synthesized using **45g** and 2-(3,4-dichlorophenyl)ethan-1-amine as starting material following General Procedure C (24%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.55-7.49 (m, 5H), 7.33 (d, J = 8.8 Hz, 1H), 7.26 (d, J = 8.8 Hz, 1H), 6.96 (d, J = 8.0 Hz, 1H), 6.76 (d, J = 4.8 Hz, 1H), 4.12 (s, 2H), 2.89 (t, J = 6.8 Hz, 2H), 2.72 (t, J = 6.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.1, 143.6, 140.2, 133.6, 132.2, 131.6, 130.6, 130.3, 130.2, 129.3, 128.1,

121.3, 120.4, 118.7, 111.9, 49.7, 43.7, 35.3; HRMS (ESI): Exact mass calcd for $C_{20}H_{17}BrCl_2N_3S$ $[M+H]^+$ 479.9704, found 479.9696; HPLC analysis: retention time = 20.9 min, peak area 95.4%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.41. 2-(3,4-Dichlorophenyl)-N-((6-(4-methoxyphenyl)imidazo[2,1-b]thiazol-5-yl)methyl)ethan-1-amine (**26**)*

Compound **26** was synthesized uisng **45h** and 2-(3,4-dichlorophenyl)ethan-1-amine as starting material following General Procedure C (49%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.54-7.49 (m, 3H), 7.30 (d, J = 7.6 Hz, 1H), 7.22 (d, J = 1.6 Hz, 1H), 6.94 (d, J = 8.8 Hz, 3H), 6.72 (d, J = 4.8 Hz, 1H), 4.11 (s, 2H), 3.84 (s, 3H), 2.86 (t, J = 6.8 Hz, 2H), 2.70 (t, J = 7.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.0, 148.8, 144.8, 140.1, 132.2, 130.6, 130.3, 130.1, 129.0, 128.1, 127.2, 119.2, 118.7, 113.9, 111.5, 55.3, 49.5, 43.6, 35.2; HRMS (ESI): Exact mass calcd for $C_{21}H_{20}Cl_2N_3OS$ $[M+H]^+$ 432.0704, found 432.0709; HPLC analysis: retention time = 9.8 min, peak area 96.4%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.42. 4-(5-(((3,4-Dichlorophenethyl)amino)methyl)imidazo[2,1-b]thiazol-6-yl)benzonitrile (**27**)*

Compound **27** was synthesized uisng **45i** and 2-(3,4-dichlorophenyl)ethan-1-amine as starting material following General Procedure C (12%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.75 (d, J = 8.4 Hz, 2H), 7.66 (d, J = 8.4 Hz, 2H), 7.49 (d, J = 4.4 Hz, 1H), 7.30 (d, J = 7.6 Hz, 1H), 7.23 (s, 1H), 6.96 (d, J = 8.8 Hz, 1H), 6.78 (d, J = 4.4 Hz, 1H), 4.13 (s, 2H), 2.88 (t, J = 6.0 Hz, 2H), 2.71 (t, J = 6.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.5, 142.7, 140.1, 139.2, 132.3, 130.6, 130.3, 130.2, 128.1, 128.0, 121.7, 119.1, 118.7, 112.6, 110.3, 49.7, 43.9, 35.4; HRMS (ESI): Exact mass calcd for $C_{21}H_{17}Cl_2N_4S$ $[M+H]^+$ 427.0551, found 427.0550; HPLC analysis: retention time = 7.1 min, peak area 95.6%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.43. N-((6-(4-(tert-Butyl)phenyl)imidazo[2,1-b]thiazol-5-yl)methyl)-2-(3,4-dichlorophenyl)ethan-1-amine (**28**)*

Compound **28** was synthesized using **45j** and 2-(3,4-dichlorophenyl)ethan-1-amine as starting material following General Procedure C (56%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.54 (d, J = 8.0 Hz, 2H), 7.49 (d, J = 4.8 Hz, 1H), 7.43 (d, J = 8.4 Hz, 2H), 7.30 (d, J = 7.6 Hz, 1H), 7.24 (d, J = 1.6 Hz, 1H), 6.94 (d, J = 8.0 Hz, 1H), 6.71 (d, J = 4.8 Hz, 1H), 4.13 (s, 2H), 2.88 (t, J = 6.8 Hz, 2H), 2.70 (t, J = 7.2 Hz, 2H), 1.35 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 150.2, 148.8, 144.8, 140.2, 132.2, 131.7, 130.6, 130.3, 130.1, 128.2, 127.5, 125.5, 119.8, 118.8, 111.5, 49.6, 43.7, 35.3, 34.6, 31.3; HRMS (ESI): Exact mass calcd for $C_{24}H_{26}Cl_2N_3S$ $[M+H]^+$ 458.1224, found 458.1233; HPLC analysis: retention time = 39.9 min, peak area 97.6%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.44. 2-(3,4-Dichlorophenyl)-N-((6-(naphthalen-2-yl)imidazo[2,1-b]thiazol-5-yl)methyl)ethan-1-amine (**29**)*

Compound **29** was synthesized using **45k** and 2-(3,4-dichlorophenyl)ethan-1-amine as starting material following General Procedure C (31%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.08 (s, 1H), 7.90-7.79 (m, 4H), 7.52-7.48 (m, 3H), 7.30-7.24 (m, 3H), 6.93 (d, J = 7.6 Hz, 1H), 6.77 (d, J = 4.0 Hz, 1H), 4.23 (s, 2H), 2.89 (t, J = 6.0 Hz, 2H), 2.71 (t, J = 6.0 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.1, 144.7, 140.3, 133.5, 132.6, 132.2, 132.1, 130.6, 130.3, 130.1, 128.1, 127.7, 126.5, 126.2, 126.02, 125.9, 120.6, 118.8, 111.8, 49.7, 43.8, 35.3; HRMS (ESI): Exact mass calcd for $C_{24}H_{20}Cl_2N_3S$ $[M+H]^+$ 452.0755, found 452.0757; HPLC analysis: retention time = 20.4 min, peak area 97.5%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.45. 6-(Naphthalen-2-yl)imidazo[2,1-b]oxazole-5-carbaldehyde (**47**)*

Compound **47** was synthesized starting using 2-bromo-1-(naphthalen-2-yl)ethan-1-one and 2-amino-oxazole as starting material following General Procedure B (50%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.98 (s, 1H), 8.28 (s, 1H), 8.00-7.89 (m, 5H), 7.58-7.55 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.5, 156.2, 139.2, 139.0, 133.8, 133.2, 129.9, 128.8, 128.6, 127.8, 127.2, 126.8, 125.9, 121.2, 114.0, 113.9.

*4.1.46. N-((6-(Naphthalen-2-yl)imidazo[2,1-b]thiazol-5-yl)methyl)-2-phenylethan-1-amine (**30**)*

Compound **30** was synthesized uisng **45k** and 2-phenylethan-1-amine as starting material following General Procedure C (42%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.01 (s, 1H), 7.90-7.80 (m, 4H), 7.55 (d, J = 4.0 Hz, 1H), 7.51-7.48 (m, 2H), 7.29-7.17 (m, 5H), 6.75 (d, J = 4.0 Hz, 1H), 4.23 (s, 2H), 2.95 (t, J = 6.8 Hz, 2H), 2.80 (t, J = 6.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.0, 144.6, 139.8, 133.5, 132.6, 132.2, 128.7, 128.5, 128.2, 128.1, 127.6, 126.4, 126.2, 126.13, 126.06, 125.8, 120.9, 118.9, 111.7, 50.2, 43.9, 36.1; HRMS (ESI): Exact mass calcd for $C_{24}H_{22}N_3S$ $[M+H]^+$ 384.1534, found 384.1534; HPLC analysis: retention time = 9.2 min, peak area 97.5%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.47. N-((6-(Naphthalen-2-yl)imidazo[2,1-b]thiazol-5-yl)methyl)-2,3-dihydro-1H-inden-2-amine (**31**)*

Compound **31** was synthesized using **45k** and 2,3-dihydro-1*H*-inden-2-amine as starting material following General Procedure C (40%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.13 (s, 1H), 7.93-7.85 (m, 4H), 7.69 (t, J = 4.8 Hz, 1H), 7.53-7.47 (m, 2H), 7.21-7.15 (m, 4H), 6.78 (d, J = 4.4 Hz, 1H), 4.29 (s, 2H), 3.71-3.66 (m, 1H), 3.16 (dd, J = 7.2 Hz, J = 8.4 Hz, 2H), 2.81 (dd, J = 5.2 Hz, J = 9.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.1, 144.5, 141.5, 133.5, 132.6, 132.2, 128.1, 127.7, 126.5, 126.4, 126.2, 126.0, 125.9, 124.7, 120.9, 118.9, 111.8, 59.1, 42.5,

40.0; HRMS (ESI): Exact mass calcd for $C_{25}H_{22}N_3S$ $[M+H]^+$ 396.1534, found 396.1529; HPLC analysis: retention time = 12.7 min, peak area 95.4%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

*4.1.48. N-((6-(Naphthalen-2-yl)imidazo[2,1-b]oxazol-5-yl)methyl)-2,3-dihydro-1H-inden-2-amine (**32**)*

Compound **32** was synthesized using **47** and 2,3-dihydro-1*H*-inden-2-amine as starting material following General Procedure C (26%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.07 (s, 1H), 7.89-7.80 (m, 5H), 7.54-7.46 (m, 3H), 7.29 (s, 1H), 7.20-7.13 (m, 5H), 4.25 (s, 2H), 3.70-3.65 (m, 1H), ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.0, 141.3, 140.7, 137.3, 137.1, 133.5, 132.5, 128.1, 128.0, 127.7, 127.6, 126.5, 126.2, 125.9, 125.8, 124.7, 116.5, 112.2, 58.9, 42.6, 39.9; HRMS (ESI): Exact mass calcd for $C_{25}H_{22}N_3O$ $[M+H]^+$ 380.1763, found 380.1767; HPLC analysis: retention time = 9.7 min, peak area 97.7%, 75:25 CH_3OH/H_2O with 0.1% NH_4OAc.

4.2. Determination of aqueous solubility

Aqueous solubility for DL5016 was determined by HPLC analysis according to a previously published protocol with minor modification [41]. Briefly, 6 mg of DL5016 was weighed and added to 1 mL of water or PBS (pH 7.4). The suspensions were shaken at 25 ^{o}C for 6 h and then centrifuged. The supernatants were filtered. Aliquots (100 μL) of the supernatants were injected into the HPLC system equipped with a C18 reverse-phase column (5 μm, 100 Å, 100×4.6 mm, Phenomenex), eluting with water-acetonitrile-triethylamine (30:70:0.01 v:v:v) for DL5016. The flow rate was 1 mL/min and the detection wavelength was set at 312 nm. The standard curve was generated by injecting 10 μL aliquots of the corresponding water or buffer solutions of DL5016 with known concentrations (Figure S2).

4.3. hCAR agonist luciferase reporter gene assay

HepG2-CYP2B6-hCAR [18] cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 5 µg/mL blasticidin (Invitrogen), 0.5 mg/mL geneticin (Invitrogen), 10% Hyclone™ FBS (GE Healthcare Life Sciences, Logan, UT), and 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen). For the assay, the HepG2-CYP2B6-hCAR cells were dispensed at 2,500 cells/4 µL/well in tissue culture–treated 1536-well white assay plates (Greiner Bio-One North America, Monroe, NC) using a Thermo Scientific Multidrop Combi (Thermo Fisher Scientific Inc., Waltham, MA). The medium used for plating was DMEM supplemented with 10% Hyclone™ FBS and 100 U/mL penicillin and 100 µg/mL streptomycin. After the assay plates were incubated at 37 ºC/5% CO_2 for 5 h, 23 nL of compounds dissolved in DMSO, CITCO (Sigma-Aldrich Corp., St. Louis, MO), or DMSO were transferred to the assay plates by a Wako Pintool station (Wako Automation, San Diego, CA). One µL of PK11195 (Sigma-Aldrich Corp.) was added (final concentration of 0.75 µM PK11195) using a Flying Reagent Dispenser (FRD, Aurora Discovery, Carlsbad, CA). The final test compound concentrations in the 5 µL assay volume ranged from 6.41 pM to 92 µM in 16 different concentrations at a 1:3 dilution. The final concentration of DMSO (used for the negative control) was 0.46%. After 23 h of incubation at 37 ºC/5% CO_2, one µL of CellTiter-Fluor™ (Promega, Madison, WI) was added, using the FRD, after which, all plates were put back into the incubator at 37 °C/5% CO_2 for another hour. The fluorescence intensity was then measured at 540 nm following excitation at 405 nm using a ViewLux plate reader (Perkin Elmer, Shelton, CT) to determine cell viability. Immediately after, 4 µL of ONE-Glo™ Luciferase reagent (Promega) was added to each well using the FRD and a 30 min incubation at room temperature occurred. Luminescence intensity was then measured using the ViewLux plate reader and data was expressed in relative luminescence units.

4.4. hCAR regulated induction of CYP2B6 and CYP3A4 in HPH

HPH obtained from Bioreclamation In Vitro Technologies (Baltimore, MD) with viability over 90% were seeded at 0.75×10^6 cells/well in 12-well biocoat plates in INVITROGRO^{TM} CP Medium (Bioreclamation In Vitro Technologies, Baltimore, MD). After attachment at 37 °C in a humidified atmosphere of 5% CO_2, hepatocytes were cultured in complete William's Medium E (WME) and overlaid with Matrigel (0.25 mg/mL). 36 h after seeding, HPH were treated with vehicle control (0.1% DMSO), CITCO (0.1, 0.5 and 1 μM), RIF (10 μM), and DL5016 (0.1, 0.5 and 1 μM) for 24 or 72 h before harvesting cells to collect RNA or protein, respectively. Total RNA isolated from HPHs was reverse transcribed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) following the manufacturers' instructions. Real-time PCR assay was performed using SYBR Green PCR Mastermix (Qiagen, Germantown, MD) on an ABI StepOnePlus real-time PCR system (Applied Biosystems). Primers for the human CYP2B6, CYP3A4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) include: CYP2B6, 5'-AGACGCCTTCAATCCTGACC-3' and 5'-CCTTCACCAAGACAAATCCGC-3'; CYP3A4, 5'-GTGGGGCTTTTATGATGGTCA-3' and 5'-GCCTCAGATTTCTCACCAACACA-3'; and GAPDH, 5'-CCCATCACCATCTTCCAGGAG-3' and 5'-GTTGTCATGGATGACCTTGGC-3'. Induction values were calculated according to the following equation: fold over control = 2ΔΔCt, where ΔCt represents the differences in cycle threshold numbers between the target gene and GAPDH, and ΔΔCt represents the relative change in these differences between control and treatment groups. Cell homogenate proteins harvested from HPH were resolved on SDS—polyacrylamide gels (4%-12%) and electrophoretically transferred onto blotting membranes. Subsequently, membranes were incubated with antibodies against CYP2B6 (diluted 1:200), CYP3A4 (diluted 1:5000), or β-actin (diluted 1:50,000). Blots

were washed and incubated with horseradish peroxidase secondary antibodies, and developed using enhanced chemiluminescence Western blotting detection reagent from GE Healthcare (Pittsburgh, PA).

4.5. hCAR nuclear translocation assay in HPH

HPH were plated in collagen-coated 96-well plates at 60,000 cells/well, media was changed 4 h after plating, and cells were infected with adenovirus-expressing enhanced yellow fluorescent protein-tagged hCAR (Ad/EYFP-hCAR; 3 μL/mL media) overnight. Subsequently, cells were treated with vehicle control (0.1% DMSO), PB (1 mM), CITCO (1 μM), or DL5016 (1 μM) for 8 h, washed with PBS, and fixed with 4% paraformaldehyde in PBS for 15 min. The nuclei of fixed cells were stained for 30 min with 4',6-diamidino-2-phenylindole (DAPI, 1 μg/mL) (Sigma-Aldrich) and five images were taken in a regular pattern for each well on a Nikon Eclipse TI fluorescent microscope for YFP and DAPI channels. General Analysis in the Nikon Elements AR High Content Analysis software package (Version 4.50.00) was used to quantify CAR translocation (%) to the nucleus by identifying the number of cells where 50% of the nucleus (DAPI) was overlapping with EYFP-hCAR and normalizing to the total number of cells expressing EYFP-hCAR (multiplied by 100%). Representative images are shown, and quantitative data represents the mean ± SD of five individual images for each treatment group.

4.6. CHOP-mediated apoptosis of SU-DHL-4 cells in the HPH/SU-DHL-4 co-culture system.

HPH were cultured in collagen-coated 12-well plates and pretreated with vehicle control (0.1% DMSO) or DL5016 (1 μM) for 24 h. The wells were then separated with 3.0 mm polycarbonate membrane inserts (Sigma-Aldrich). A total of 0.5×10^6 SU-DHL-4 cells suspended in supplemented Williams' Medium E were transferred into the insert chamber with a final volume

of 2 mL/well. The co-cultures were exposed to designated concentrations of the chemotherapy drugs in CHOP in the presence or absence of DL5016 for various time intervals, as indicated. The viability of the SU-DHL-4 cells was determined at selected time points with a Cellometer Auto T4 (Nexcelom Biosciences) using trypan blue exclusion.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at xxx

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Highlights

- DL5016 activates hCAR with excellent potency (EC_{50} = 0.66 μM, E_{MAX} = 4.9)
- DL5016 robustly induces the expression of hCAR target genes CYP2B6, at both the mRNA and protein levels
- DL5016 dramatically enhances the efficacy of CPA-based chemotherapeutics CHOP.