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Exploring the structure activity relationship and mechanism of a chromene scaffold (CXL series) for its selective anti-proliferative activity towards multidrug resistant cancer cells

Tengfei Bian,[1] Kavitha Chandagirikoppal Vijendra,[1,3] Yi Wang,[1] Amy Meacham,[4,5] Santanu Hati,[1] Christopher R. Cogle,[4,5] Haifeng Sun,[1,2,*] Chengguo Xing[1,2,4,*]

[1]Department of Medicinal Chemistry, College of Pharmacy, University of Florida, 1345 Center Drive, Gainesville, FL 32610

[2]Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455

[3]Department of Chemistry, Government College for Women Mandya, University of Mysore, Karnataka, INDIA-571 401

[4]University of Florida Health Cancer Center, Gainesville FL 32610

[5]Division of Hematology and Oncology, Department of Medicine, College of Medicine, University of Florida, Gainesville FL 32610

Abstract

Multiple drug resistance (MDR) is one major barrier in cancer management, urging for new drugs to help treat MDR malignancies and elucidate MDR mechanisms. A series of chromene compounds (CXL series) demonstrate increased anti-proliferative activity towards MDR acute myeloid leukemia (AML) cells. Structure-activity relationship (SAR) on anti-proliferative potency has been partly characterized while the structural determinants contributing to its selectivity have not been investigated. In this study, three series of CXL compounds were synthesized and evaluated in HL60 and HL60/MX2 leukemia cells. The results not only confirmed previous SAR but also for the first time provided structural insights for its selectivity in MDR HL60/MX2 cells. Using the lead compounds as probes, we demonstrated that modulating intracellular calcium homeostasis is responsible for their anti-proliferative potency and selectivity. Three candidates also demonstrate excellent *in vitro* safety profiles between cancer cells and normal cells, which will be evaluated *in vivo* in future studies.

Introduction

Most anti-neoplastic agents only achieved modest success, in part due to the rapid development of multi-drug resistance (MDR). Therefore, there is an unmet and urgent need for new anticancer drugs that can selectively target MDR cancers to improve clinical outcomes.

Cancer cells acquire MDR via multiple mechanisms.[1-6] One major mechanism is the overexpression of ATP-binding cassette (ABC) transporter proteins, such as p-glycoprotein.[7,8] ABC transporter proteins export various substrates across the cellular membranes, including certain anticancer drugs, resulting in decreased intracellular drug concentrations and thereby conferring MDR. Another common mechanism is the resistance to apoptosis.[9-11] Cancer cells typically overexpress the anti-apoptotic Bcl-2 family proteins that introduce resistance to apoptosis induced by conventional chemotherapeutic agents. Inhibiting the anti-apoptotic Bcl-2 family proteins therefore is a possible strategy to treat MDR cancer.[12,13] Bcl-2 family proteins can locate on mitochondria and endoplasmic reticulum (ER).[14] Inhibitors designed to target mitochondrial anti-apoptotic Bcl-2 family proteins have demonstrated some success to nullify MDR.[15,16] Bcl-2 family proteins on ER has not been investigated much for their therapeutic opportunities.[14] The ER located Bcl-2 family proteins regulate apoptosis in part via modulating calcium homeostasis with ER calcium transporters, such as sarco endoplasmic reticulum ATPase (SERCA)[17-19] and inositol triphosphate receptors (IP_3Rs).[20,21] Calcium, an important secondary messenger, is involved in the regulation of a wide range of cellular processes, including different types of cell death.[22-24] Interestingly intracellular calcium perturbation has been reported in MDR cancer cells,[25,26] in addition to its regulations and communications of the ABC transporter proteins[27] and the Bcl-2 family proteins.[28] Therefore the ABC transporter protein, the Bcl-2

family protein, ER calcium transporters and intracellular calcium may be interrelated in MDR.[29] Compounds that modulate these processes may lead to viable therapeutics to effectively manage MDR in cancer treatment.

We previously discovered a chromene-based compound – sHA 14-1 (**1**, Figure 1),[30] derived from an early unstable putative Bcl-2 inhibitor – HA 14-1 (**2**, Figure 1).[31] Genetic overexpression of anti-apoptotic Bcl-2 or Bcl-xL proteins failed to confer resistance to sHA 14-1 although such overexpression successfully induced MDR to conventional cancer therapies.[30] Mechanistically, sHA 14-1 treatment led to a rapid but moderate cytosolic calcium increase from the ER calcium store with the induction of ER stress followed by the mitochondrial apoptotic process, suggesting that ER may be the up-stream organelle for its anti-proliferative activity with calcium as the potential signaling messenger.[32] Later SAR studies of sHA 14-1, termed CXL series of compounds, characterized the structural features important to the anti-proliferative activity.[33-36] Interestingly, multiple naturally acquired MDR cancer cells were found to be more sensitive to these compounds relative to their parental cells, which differentiate them from the conventional therapeutic agents. Furthermore, cancer cells failed to acquire resistance to CXL compounds even upon chronic exposure, as demonstrated by CXL017 in both HL60 and HL60/MX2.[37] These unique and important features support their potential in addressing MDR in cancer treatment. Because the discovery and optimization of CXL series have been dictated by phenotype-based screening for candidates to selectively target MDR cancer cells, the up-stream mechanisms for its anti-proliferative activity and selectivity remain to be fully characterized. Nevertheless, anti-apoptotic Bcl-2 family protein, SERCA, ER calcium and ER stress appeared

to be perturbed by CXL compounds,[32,37,38] which may account for its ability to overcome MDR in cancer cells.

In this study, we synthesized three new sets of CXL compounds and characterized the structural determinants critical for their selective anti-proliferative activity towards MDR cancers. Given that the induction of cytosolic calcium increase is rapid,[32,38] which may be the up-stream signaling, the importance of intracellular calcium homeostasis in two pairs of MDR cancer cells and in the selective anti-proliferative activity of CXL series were investigated. Finally, three CXL lead candidates were identified with excellent therapeutic windows for future translational development.

Results and Discussion

SAR in HL60 and HL60/MX2 cells The results of our previous SAR studies showed that the aromatic substitutions at the 6-position of the chromene was the most optimal while subtle changes on the aromatic ring could result in significant changes in the anti-proliferative potency.[33,35] It was also found that the two propargyl esters were critical for potency that replacing the propargyl with a wide range of functional groups led to a significant loss of activity while replacing the ester also compromised the potency.[34,36] Therefore, we focused our structural modifications on the aromatic substitutions in this study. Since our earlier phenyl-based lead compounds suffered from poor water solubility, pyridine and other heterocyclic aromatic functional groups were explored herein. We evaluated the impact of polarity, electronegativity, rigidity and size of the substituents on both anti-proliferative activity and selectivity. The first series of compounds were designed to contain a pyridine via a para connection to the 6-position

of the chromene scaffold (Table 1, **6a – 6w**). Most of these compounds have mono substitution at the ortho position of the pyridine, except for CXL143 (**6j**) which has dimethyl substituents at both ortho positions. The second series of compounds were designed to contain a pyridine via a meta connection to the 6-position of the chromene scaffold with mono substituents on the pyridine ring, meta or para to the 6-positin of the chromene (Table 2, **6x – 6ae**). The third series preliminarily explored the ring size (Table 3, **6af – 6ah**). The syntheses of these compounds follow the general procedures as delineated in Scheme 1. The most challenging step was the final step, which involved the base-aided addition, ring opening, ring cyclization, and esterification with ethyl cyanoacetate in propargyl alcohol. This reaction was highly moisture sensitive that the inclusion of freshly dried molecular sieves was essential and the yield varied significantly with different substrates. The other steps are generally smooth with good to excellent yields. In brief, 4-bromo coumarin (**3**) was prepared from salicylaldehyde with N,N-dimethylacetamide via cyclization under $POCl_3$ treatment. The boronic acid pinacol ester group was introduced by the coupling reaction catalyzed by $Pd(dppf)Cl_2$ with potassium acetate as the base to generate intermediate **4**. Most of the intermediates **5** were synthesized through the Suzuki coupling reaction under the standard conditions. The final products (**6**) were obtained from intermediate **5** upon the treatment of sodium, propargyl alcohol and ethyl cyanoacetate. **6m** and **6n** were prepared from the corresponding intermediate **5** by standard de-esterification, amidation and the final step. **6r** was prepared from **6b** via a standard amidation with glycolic acid.

Twenty three analogs have been prepared in the first series (Table 1). Consistent with previous SAR results,[34,35] small to medium sized substituents on the meta position of the pyridine were preferred with respect to anti-proliferative activity, such as **6f**, **6s**, **6u** while no

substituent **6a**, a fluoro substitution **6d** or large substituents (**6l**, **6n**, and **6v**) were not optimal. There are exceptions. For instance, a cyano substituent (**6e**) was not favorable, potentially due to its rigidity while **6q** and **6r** showed decent potency. There was no clear correlation of anti-proliferative activity with respect to polarity and electronegativity, suggesting that these parameters are less important relative to the size of the substituents. Nevertheless, eight compounds showed submicromolar anti-proliferative activity in HL60/MX2 cells with **6f** being the most potent. In addition, the anti-proliferative activities of **6f** and **6a** differed by 74-fold and 53-fold in HL60/MX2 and HL60 cells respectively despite their similar chemical structure and physicochemical property. The second series had eight compounds (Table 2). Mono substituents were introduced either meta or para to the chromene scaffold. It appeared that substitution para to the chromene scaffold resulted in candidates of weaker potency (**6x** vs **6ad**, **6y** vs **6ab** and even **6z** vs **6aa**), consistent with our previous observations that the position para to the chromene do not favor any modifications. Similar to the first series, a large substituent was not favored as well (**6ae**). In this series, the anti-proliferative activities of **6y** and **6ab** differed by 41-fold and 58-fold in HL60/MX2 and HL60 cells respectively despite their similar chemical structure and physicochemical property. For the last series, three non-pyridine heteroaromatic substituents were explored (Table 3). Again, a large substituent was not tolerated (**6ah**). Similarly, two 1-methylpyrazole analogs (**6af** and **6ag**) only have slight structural differences but showed significant different anti-proliferative activities with a 16-fold and 34-fold differences in HL60/MX2 and HL60 respectively. **6af** has the most potent anti-proliferative activity among the CXL series discovered to date. In summary, the SAR with respect to the potency of anti-proliferative activity observed herein overall was consistent with our previous results that

the position on the aromatic ring para to the chromene does not favor modifications, likely due to steric effect. The meta position favors mono substituents of small to medium size with structural flexibility. Three pairs of CXL compounds (**6a** vs. **6f**, **6y** vs. **6ab**, and **6af** vs. **6ag**) may serve as chemical probes to investigate their anti-proliferative mechanisms because of high structural similarity but distinct potency.

The selectivity of these analogs towards MDR resistant cell lines was determined based on the ratio of their GI_{50}s in HL60 cells to that in HL60/MX2 cells – the larger the ratio, the compound is the more selective towards MDR cancer cells. We classified these compounds into two categories based on their selectivity ratio and statistical analysis. Compounds with a selectivity ratio < 2 typically had no significant difference in their GI_{50}s in HL60 and HL60/MX2 ($p > 0.05$), indicating that they are non-selective towards MDR cancer cells. Analogs with a selectivity ratio > 2 all had a significant difference in their GI_{50}s ($p < 0.05$) and were considered selective towards MDR cancer cells. Clear SAR was observed on selectivity for the first time – analogs with larger substituents in all three series of compounds had low selectivity with no exception, including **6h**, **6l**, **6n**, **6q**, **6r**, **6t**, **6v**, **6w**, **6ae**, and **6ah** (Tables 1 – 3, which are shaded). Indeed MDR HL60/MX2 showed resistance to some of these compounds with large substituents, such as **6ae** and **6ah**. On the other hand, analogs with smaller substituents or no substituents all demonstrated selective anti-proliferative activity towards MDR HL60/MX2 cells. Most of these compounds showed 2 – 4 fold selectivity and a few of them revealed higher selectivity, such as **6d**, **6z**, **6ac**, and **6ag**. The best examples to further elaborate the SAR on selectivity is **6ad** and **6ae**, which differ from each other only on the R^4 substituents from an unsubstituted amine to an acetamide. Both compounds had similar anti-proliferative activities in HL60 cells while the GI_{50} of **6ad** in HL60/MX2 was

34-fold more potent than **6ae**. We envision that **6ae** can be paired with **6ad** as chemical probes to investigate the mechanism responsible for their significant anti-proliferative differences in HL60/MX2 cells with HL60 cells as the control, which may reveal the molecular basis for MDR and the mechanism of the selectivity of the CXL compounds.

There were no clear correlations between the selectivity and anti-proliferative activity of the CXL compounds in HL60 and HL60/MX2. Compounds of the best selectivity typically had moderate anti-proliferative potency, such as **6d**, **6z**, **6ac**, and **6ag**. The most potent candidates typically have a 2 – 3 fold selectivity towards HL60/MX2, including **6f**, **6ab**, and **6af**. It remains to be determined whether candidates of improved potency and selectivity may be developed via further structural modifications. It is also possible that the above lead candidates may be potent and selective enough for future translational evaluation.

Characterization of the MDR phenotype and CXL selectivity in an isogenic pair of ovarian cancer cell lines – OVCAR-8 and NCI/ADR-RES To date, CXL compounds have been evaluated in AML and other leukemia cell lines, demonstrating selective anti-proliferative profile towards MDR cancer cells.[34] **6f** also demonstrated selectivity towards three additional hematological MDR cells (in preparation of publication separately), consistent with our previous results.[34] On the other hand, the selective anti-proliferative nature of CXL in MDR cancer cell lines derived from solid tumors remains to be evaluated. To explore this, we obtained and characterized two isogenic ovarian cancer cells from National Cancer Institute (NCI) – OVCAR-8 and NCI/ADR-RES. NCI/ADR-RES was initially considered as a drug-resistant breast cancer cell line upon chronic doxorubicin challenge until it was authenticated to derive from OVCAR-8.[39]

We first characterized the MDR phenotype nature of NCI/ADR-RES relative to OVCAR-8 by evaluating their sensitivities to cisplatin, doxorubicin, and taxol following the 48-h anti-proliferative assay (Figure 2). NCI/ADR-RES showed significantly reduced sensitivity to doxorubicin, consistent with the chronic exposure to doxorubicin during its development. It also showed significant resistance to taxol and moderate resistance to cisplatin. These data overall demonstrate the MDR phenotype of NCI/ADR-RES relative to OVCAR-8. **6f**, **6ab**, and **6af**, the most potent anti-proliferative candidates in the three series of CXL compounds with moderate selectivity, were evaluated for their anti-proliferative activity in both OVCAR-8 and NCI/ADR-RES cells. In all concentrations tested, **6f** and **6ab** showed higher anti-proliferative activity in NCI/ADR-RES cells relative to OVCAR-8 cells. For **6af**, it also showed higher anti-proliferative activity to NCI/ADR-RES cells at the lowest concentration. Two higher concentrations of **6af** did not show selectivity, potentially because the higher concentrations significantly inhibited the cell growth, leaving less window to detect the selectivity. These ovarian cancer data in combination with the results in HL60 and HL60/MX2 cells support the potential scope of the CXL series to selectively target cancers with MDR phenotype.

Characterization of the proliferation rate and ER calcium content of the isogenic parental and MDR cancer cells Stimulated by the selective anti-proliferative activity of CXL series towards HL60/MX2 and NCI/ADR-RES, we first explored whether such a selectivity was due to the different rates of proliferation between HL60 and HL60/MX2, and between OVCAR-8 and NCI/ADR-RES. In another word, whether HL60/MX2 and NCI/ADR-RES have higher proliferation rates than the parental cell lines that endow their increased sensitivity to CXL

compounds. These were performed by seeding the same number of parental and MDR cells at the optimal density. After 48-h culturing under the standard conditions, the number of cells were determined via cell counting. HL60/MX2 has a higher proliferation rate than HL60 while NCI/ARD-RES has a reduced proliferation rate than OVCAR-8 (Figure 3). These results demonstrate that the proliferation rate differences between the isogenic cells are not likely the key mechanism responsible for CXL's selective anti-proliferative activity towards the MDR cells.

As discussed, the intracellular calcium, as a second messenger, is involved in the regulation of various molecular processes, including proliferation, cell death and MDR. Since our earlier work showed that CXL compounds perturbs the intracellular calcium of cancer cells,[28,32,33] this may be mechanistically responsible for its selective anti-proliferative activity towards MDR cancer cells. We therefore quantified the intracellular ER calcium content in the parental and MDR cancer cells. Briefly, the cells were pre-loaded with an ester of Fluo-4 for 30 min. Upon maintaining the cells in calcium free medium, cells were exposed to thapsigargin, a SERCA inhibitor that inhibits ER calcium reuptake. This led to the depletion of ER calcium and the increase in cytosolic calcium. The cytosolic calcium, upon binding to Fluo-4, resulted in increased fluorescence. Carrier treatment, ionomycin treatment in calcium free medium and ionomycin treatment in calcium containing medium provided the baseline, the total intracellular calcium and the maximum fluorescence signal of Fluo-4 with saturated calcium. These were used to determine the cytosolic calcium concentration as detailed in the Experimental section, which reflected the ER calcium content. Both HL60/MX2 and NCI/ADR-RES had higher ER calcium contents in comparison to their isogenic parental controls (Figure 4). There also

appeared to be a higher increase of intracellular calcium in HL60/MX2 relative to HL60 (52%) in comparison to NCI/ADR-RES relative to OVCAR-8 (22%), consistent with the data that CXL compounds showed higher selectivity towards HL60/MX2 relative to HL60 in comparison to NCI/ADR-RES relative to OVCAR-8.

Next, we evaluated whether CXL compound treatment perturbed intracellular calcium homeostasis and whether the potency in perturbing intracellular calcium homeostasis had any correlation with their anti-proliferative activity and selectivity. In this study, HL60 and HL60/MX2 were used for evaluation because of our extensive anti-proliferative evaluation of the CXL series in these cells (Tables 1 – 3), the relatively larger anti-proliferative selectivity of CXL in these cells, and the bigger differences in their ER calcium content (Figure 4). Two pairs of compounds were evaluated because of their high structural similarity and significant differences in anti-proliferative activity – **6f** vs **6a** and **6ab** vs **6y**. These compounds were evaluated at the concentrations of 1 μM and 5 μM respectively because these concentrations were in between the GI_{50} of the potent analogs (**6f** and **6ab**) and the less active controls (**6a** and **6y**). The experiments followed the same procedures as above for intracellular calcium quantification except that CXL compounds were used instead of thapsigargin. The cytosolic calcium increase was the difference between CXL treatment and DMSO treatment. In both cell lines, the potent compounds (**6f** and **6ab**) at both concentrations induced more cytosolic calcium increase in comparison to their corresponding negative control compounds, although some of the differences were not statistically significant (Figure 5).

We next explore whether the increased cytosolic calcium may be the responsible signal for the anti-proliferative activity of CXL compounds. In this direction, we evaluated whether

chelating the cytosolic calcium could protect the cells from CXL compounds. Briefly, cells were pre-treated with 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA, a calcium chelator) acetoxymethyl ester (2.5 μM) or DMSO carrier for one hour. Such cells were then treated with **6f** with its anti-proliferative activity determined (Figure 6). Upon BAPTA treatment, both HL60 and HL60/MX2 gained resistance to **6f**. Overall the extent of protection appears to be more significant in HL60 in comparison to HL60/MX2 cells. This is potentially because HL60/MX2 has higher ER calcium content than HL60 (Figure 4) that more cytosolic calcium increase would be introduced upon **6f** treatment in HL60/MX2 cells relative to HL60 cells so that the chelating effect by BAPTA was not as significant as that in HL60 cells.

These results suggest that rate of proliferation is not the mechanism for the selective anti-proliferative activity of CXL compounds towards MDR cancer cells while inducing cytosolic calcium increase from ER is at least part of the mechanism responsible for its anti-proliferative activity and selectivity.

Selectivity of **6f**, **6ab** and **6af** towards cord blood cells and bone marrow cells in comparison to cytarabine and mitoxantrone We next evaluated the potential therapeutic windows of three CXL lead compounds between cancer cells and normal cells. Given that most of our evaluations focused on AML, we selected umbilical cord (UBC) blood cells and adult bone marrow (BM) cells as normal cells for comparison. Cytarabine and mitoxantrone were selected as the conventional chemotherapy controls given their wide use in the clinical setting for AML and other hematological malignancies. CXL treatment of three normal specimens (two UBC units and one BM donor) showed no toxicity with GI_{50} values > 100 μM, in comparison to the GI_{50}

values in both parental HL60 and MDR HL60/MX2 cells in the mid to high nM range (Table 4). These results showed at least 200 – 1000 fold selective anti-proliferative activity of CXL compounds towards AML cells, particularly MDR AML cells, compared to normal hematopoietic cells. Although the tested concentrations of cytarabine did not produce toxicity in UBC, it showed weak toxicity in BM cells, which resulted in a relatively narrow selectivity. Mitoxantrone on the other hand showed weak toxicity to UBC cells and significant toxicity to BM cells. Even though mitoxantrone was more potent to HL60 than all three CXL candidates, mitoxantrone was much less potent in HL60/MX2 cells. The therapeutic window of mitoxantrone in HL60/MX2 cells and BM cells was only 13 fold while the least therapeutic window for CXL compounds between HL60/MX2 and BM cells was over 500 fold. These results reveal the optimal selective anti-proliferative activity of CXL series toward cancer cells with promising therapeutic windows, particularly towards cancer cells with MDR phenotype.

Conclusions

In this study, three new series of chromene compounds (CXL series) were synthesized and their anti-proliferative activity examined in AML and ovarian cancer cells and the corresponding MDR counter parts. The SAR results confirmed the structural determinants of CXL compound series important to potency. Furthermore a clear SAR was observed for CXL's selective anti-proliferative activity towards MDR cancers – the size of the substituents was the most critical such that small substituents were necessary while large substitutions led to decrease or complete loss in selectivity. Mechanistically, ER calcium homeostasis appeared to be different in parental and MDR cancer cells. MDR cells showed higher ER calcium content and CXL

treatment reduced ER calcium and increased cytosolic calcium, which was at least partly responsible for its anti-proliferative potency and potentially selectivity. Three lead CXL compounds of mid to high nM potency, demonstrating excellent selectivity between normal cells and cancer cells as well as MDR counterparts, are being developed for future clinical oncology applications.

Experimental Section

Chemicals and Reagents. Fluo-4AM Direct™ Calcium assay kits was obtained from Invitrogen. Thapsigargin, ionomycin calcium salt and BAPTA were obtained from Sigma-Aldrich. CellTiter-Blue Assay Kit was obtained from Promega.

Chemistry. All reagents and solvents were purchased from vendors and used without further purification or distillation unless otherwise stated. Analytical thin layer chromatography was performed on Whatman silica gel 60 Å with fluorescent indicator (Partisil K6F). Compounds were visualized by UV light and/or stained with potassium permanganate solution followed by heating. Flash column chromatography was performed on Whatman silica gel 60 Å (230-400 mesh). NMR (^{1}H and ^{13}C) spectra were recorded on a Bruker 500 MHz spectrometer and calibrated using an internal reference. All compounds synthesized are racemic mixtures and are more than 95% pure based on HPLC analysis. The characterizations of the intermediates were included in the Supporting Materials with representatives included herein while the characterizations of all final compounds were included herein.

General synthetic procedures To 2.8 mL N, N-dimethylacetamide (30.0 mmol), stirred at 0 □, was added 2.8 mL phosphorus oxychloride (30.0 mmol) slowly. The reaction mixture was stirred at 0 □ for 30 min followed by the addition of 5-bromosalicylaldehyde (3.0 g, 15 mmol) in 15 mL dry dichloromethane. The solution was then refluxed for 30 min. 150 mL $NaHCO_3$ saturated solution was added to the cooled reaction mixture, and the mixture was heated at 80 □ for 30 min. After acidification with 1N HCl, the mixture was extracted with dichloromethane, dried over $MgSO_4$, and concentrated under reduced pressure to give the crude product. Compound **3** was obtained via crystallization in ethanol as slightly yellow solid with 76% yield.

To a slurry of 6-bromocoumarin (**3**, 4.48 g, 20 mmol), bis(pinocolato)diboron (7.62g, 30 mmol), and potassium acetate (7.85 g, 80 mmol) in anhydrous dioxane (100 mL) was added (1,1-Bis(diphenylphosphino)ferrocene) palladium dichloride (1.47 g, 2 mmol). The mixture was stirred at 80 □ for 3 hours under an inert N_2 atmosphere. The reaction mixture was poured into water and extracted with ethyl acetate (120 mL). The combined organic phase was washed with saturated sodium chloride, dried over $MgSO_4$, and concentrated to give the crude product. The pure product (**4**) was obtained through silica gel chromatography as a white solid. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.96−7.94 (m, 2H), 7.72 (d, J = 9.5 Hz, 1H), 7.32 (d, J = 9.0 Hz, 1H), 6.42 (d, J = 9.5 Hz, 1H), 1.36 (s, 12H).

$Pd(PPh_3)_4$ (58 mg, 0.05 mmol) was added to the solution of 2-amino-4-bromopyridine (157 mg, 1 mmol), and the boron ester (**4**, 272 mg, 1 mmol) in 8 mL dimethoxyethane followed by the addition of 2M Na_2CO_3 (made in-house, 1.5 mL) in a two-neck flask. The reaction mixture was vacuumed and filled N_2 back three times before being heated at 80 □ for 3 hours. 15 mL water and 15 mL ethyl acetate were added separately to the cooled reaction mixture. The water phase was extracted by another 30 mL ethyl acetate twice, and the combined organic solution was washed with saturated NaCl, dried over $MgSO_4$ and concentrated under reduced pressure to give the crude product, which was purified through silica gel chromatography to give compound **5** as a white solid with a 78% yield. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.15 (d, J = 5.5 Hz, 1H), 7.77-7.32 (m, 2H), 7.69 (d, J = 1.5 Hz, 1H), 7.41 (d, J = 8.5 Hz, 1H), 6.86 (d, J = 5.5 Hz, 1H), 6.69 (s, 1H), 6.49 (d, J = 9.5 Hz, 1H), 4.58 (s, 2H).

Two small pieces (1 mmol) of freshly cut sodium was added to a slurry of 7 mL propargyl alcohol (dried before used) and 8 gram freshly activated molecular sieves, followed by the

addition of ethyl cyanoacetate (1 mmol). The mixture was stirred at room temperature under N_2 for 30 min, followed by the addition of a solution of the corresponding coumarin (**5**, 0.4 mmol) in propargyl alcohol (5 mL). The resulting reaction mixture was stirred at room temperature. Upon the consumption of the coumarin (24 – 36 hours), the reaction mixture was concentrated, diluted with water (30 mL), and extracted with methylene chloride (3 x 20 mL). The organic fractions were combined, washed with saturated NaCl, dried over $MgSO_4$, and the solvent was removed under reduced pressure to afford an oil. The pure compound was obtained from the crude oil via silica gel chromatography.

Prop-2-yn-1-yl 2-amino-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-6-(pyridin-4-yl)-4*H*-chromene-3-carboxylate (CXL131) (6a). Slightly yellow solid, yield 82%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.64 (d, *J* = 5.5 Hz, 2H), 7.58 (s, 1H), 7.50-7.46 (m, 3H), 7.09 (d, *J* = 8.5 Hz, 1H), 6.45 (brs, 2H), 4.84-4.77 (m, 2H), 4.64-4.57 (m, 2H), 4.42 (dd, *J* = 7.5, 4.5 Hz, 1H), 2.77 (dd, *J* = 15.5, 4.5 Hz, 1H), 2.70 (dd, *J* = 15.5 Hz, 4.5 Hz, 1H), 2.48 (s, 1H), 2.30 (s, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.6, 167.8, 162.0, 150.6, 150.3, 147.3, 134.6, 127.2, 126.6, 126.0, 121.4, 116.8, 78.6, 77.5, 75.8, 74.8, 74.4, 51.9, 51.2, 43.3, 31.0. HRMS (ESI+) m/z calcd for $C_{23}H_{19}N_2O_5$ $[M+H]^+$, 403.1288, found 403.1278.

Prop-2-yn-1-yl 2-amino-6-(2-aminopyridin-4-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL132) (6b). Slightly yellow solid, yield 84%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.11 (d, *J* = 5.5 Hz, 1H), 7.71 (d, *J* = 1.5 Hz, 1H), 7.63 (dd, *J* = 8.5, 2.0 Hz, 1H), 7.55 (d, *J* = 8.5 Hz, 1H), 6.83 (dd, *J* = 5.5, 1.0 Hz, 1H), 6.66 (s, 1H), 6.63 (brs, 2H), 4.80-4.76 (m, 2H), 4,64-4.56 (m,

2H), 4.50 (s, 2H), 4.40 (dd, *J* = 7.5, 4.0 Hz, 1H), 2.76 (dd, *J* = 15.5, 4.5 Hz, 1H), 2.78 (dd, *J* = 15.5, 2.5 Hz, 1H), 2.34 (t, *J* = 2.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.6, 167.8, 166.0, 158.9, 157.3, 149.2, 148.6, 145.3, 137.1, 136.5, 125.7, 116.5, 112.6, 106.0, 78.6, 77.6, 75.9, 74.9, 74.3, 51.9, 51.2, 74.3, 51.9, 51.2, 43.3, 31.0. HRMS (ESI+) m/z calcd for $C_{23}H_{20}N_3O_5$ $[M+H]^+$, 418.1397, found 418.1386.

Prop-2-yn-1-yl 2-amino-6-(2-hydroxypyridin-4-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL133) (6c). White solid, yield 53%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.20 (d, *J* = 7.0 Hz, 1H), 6.81 (d, *J* = 2.0 Hz, 1H), 6.36 (dd, *J* = 7.0, 2.0 Hz, 1H), 4.08-4.06 (m, 2H), 3.91 (t, *J* = 5.0 Hz, 2H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 173.0, 167.2, 159.7, 156.0, 150.9, 148.9, 139.8, 137.3, 129.7, 129.6, 126.9, 117.6, 101.3, 99.8, 88.2, 78.7, 78.6, 76.9, 76.8, 55.6, 51.4, 41.0, 30.1. HRMS (ESI+) m/z calcd for $C_{23}H_{19}N_2O_6$ $[M+H]^+$, 419.1238, found 419.1224.

Prop-2-yn-1-yl 2-amino-6-(2-fluoropyridin-4-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL164) (6d). Slightly yellow oil, yield 74%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.25 (s, *J* = 5.0 Hz, 1H), 7.58 (d, *J* = 2.0 Hz, 1H), 7.35 (dd, *J* = 7.0 Hz, 2.0 Hz, 1H), 7.11-7.09 (m, 2H), 4.81-4.79 (m, 2H), 4.66-4.57 (m, 2H), 4.42 (dd, *J* = 7.5 Hz, 4.0 Hz, 1H), 2.77 (dd, *J* = 15.5 Hz, 4.0 Hz, 1H), 2.70 (dd, *J* = 16.0 Hz, 8.0 Hz, 1H), 2.48 (t, *J* = 2.5 Hz, 1H), 2.35 (t, *J* = 2.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.6, 167.7, 165.8, 165.5, 161.9, 153.0, 152.9, 150.4, 148.0, 133.5, 133.4, 127.4, 126.7, 126.2, 119.5, 119.3, 116.9, 107.0, 106.7, 78.7, 77.6, 75.8, 74.9, 74.4, 51.8, 51.3, 43.3, 30.9. HRMS (ESI+) m/z calcd for $C_{23}H_{18}FN_2O_5$ $[M+H]^+$, 421.1194, found 421.1192.

Prop-2-yn-1-yl 2-amino-6-(2-cyanopyridin-4-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carb

oxylate (CXL144) (6e). White solid, yield 14%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.73 (d, *J* = 5.0 Hz, 1H), 7.87 (s, 1H), 7.67 (d, *J* = 1.5 Hz, 1H), 7.61 (d, *J* = 1.5 Hz, 1H), 7.49 (dd, *J* = 8.5 Hz, 2.0 Hz, 1H), 7.13 (d, *J* = 8.5 Hz, 1H), 4.84-4.75 (m, 2H), 4.66-4.58 (m, 2H), 4.43 (dd, *J* = 7.5, 4.0 Hz, 1H), 2.78 (dd, *J* = 15.5, 4.5 Hz, 1H), 2.70 (dd, *J* = 15.5, 4.5 Hz, 1H), 2.48 (s, 1H), 2.37 (s, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.6, 167.6, 161.7, 151.5, 151.4, 148.7, 134.6, 132.3, 127.5, 126.7, 126.6, 126.2, 124.3, 117.3, 117.2, 78.5, 77.5, 75.7, 74.9, 74.4, 51.9, 51.3, 43.2, 30.9, 29.7. HRMS (ESI+) m/z calcd for $C_{24}H_{18}N_3O_5$ $[M+H]^+$, 428.1241, found 428.1227.

Prop-2-yn-1-yl 2-amino-6-(2-methylpyridin-4-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL146) (6f). White solid, yield 25%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.53 (d, *J* = 5.5 Hz, 1H), 7.55 (d, *J* = 2.0 Hz, 1H), 7.47 (d, *J* = 2.0 Hz, 1H), 7.33-7.26 (m, 2H), 7.08 (d, *J*= 8.5 Hz, 1H), 6.42 (brs, 2H), 4.80-4.76 (m, 2H), 4.61-5.93 (m, 2H), 4.41 (dd, *J* = 7.5, 4.5 Hz, 1H), 2.77 (dd, *J* = 15.5, 4.5 Hz, 1H), 2.71 (dd, *J* = 15.5, 4.5 Hz), 2.62 (s, 3H), 2.48 (t, *J* = 2.5 Hz, 1H), 2.31 (t, *J* = 2.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.6, 167.8, 162.0, 158.9, 150.5, 149.6, 147.6, 134.9, 127.2, 126.6, 125.9, 120.9, 118.6, 116.6, 78.6, 77.5, 75.8, 74.8, 74.4, 51.9, 51.2, 43.3, 31.0, 24.6. HRMS (ESI+) m/z calcd for $C_{24}H_{21}N_2O_5$ $[M+H]^+$, 417.1445, found 417.1430.

Prop-2-yn-1-yl 2-amino-6-(2-(hydroxymethyl)pyridin-4-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL137) (6g). Slightly yellow solid, yield 78%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.58 (d, *J* = 5.0 Hz, 1H), 7.58 (d, *J* = 2.0 Hz, 1H), 7.49 (dd, *J* = 6.5, 2.0 Hz, 1H), 7.43 (s, 1H), 7.38 (d, *J* = 5.0 Hz, 1H), 7.09 (d, 8.5 Hz, 1H), 6.42 (brs, 2H), 4.80 (s, 2H), 4.64-4.56 (m, 2H), 4.42 (dd, *J* = 7.5, 4.0 Hz, 1H), 2.77 (dd, *J* = 15.5, 4.0 Hz, 1H), 2.70 (dd, *J* = 15.5, 7.5 Hz,

1H)，2.48 (t, *J* = 2.5 Hz, 1H), 2.32 (t, *J* = 2.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.6, 167.8, 162.0, 159.7, 150.7, 149.0, 148.1, 139.5, 137.3, 136.7, 126.1, 120.3, 118.1, 116.8, 77.6, 75.8, 74.9, 74.4, 64.3, 51.9, 51.3, 43.3, 31.0. HRMS (ESI+) m/z calcd for $C_{24}H_{21}N_3O_6$ $[M+H]^+$, 433.1394, found 433.1381.

Prop-2-yn-1-yl 2-amino-6-(2-(methoxymethyl)pyridin-4-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL149) (6h). Slightly yellow solid, yield 67%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.58 (d, *J* = 5.0 Hz, 1H), 7.59 (d, *J* = 8.5 Hz, 1H), 7.51 (dd, *J* = 7.5 Hz, 2.0 Hz, 1H), 7.36 (dd, *J* = 5.0 Hz, 1.5 Hz, 1H), 7.08 (d, *J* = 8.5 Hz, 1H), 6.45 (brs, 2H), 4.82-4.80 (m, 2H), 4.65-4.60 (m, 4H), 4.42 (dd, *J* = 7.5 Hz, 4.5 Hz, 1H), 2.77 (dd, *J* = 15.5 Hz, 4.5 Hz, 1H), 2.71 (dd, *J* = 15.5 Hz, 7.5 Hz, 1H), 2.48 (t, *J* = 2.5 Hz, 1H), 2.35 (t, *J* = 2.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.6, 167.8, 159.0, 150.6, 149.6, 148.0, 134.7, 127.2, 126.7, 126.0, 120.1, 118.9, 77.6, 75.8, 75.5, 74.9, 74.4, 58.9, 51.9, 51.2, 43.3, 31.1. HRMS (ESI+) m/z calcd for $C_{25}H_{23}N_2O_5$ $[M+H]^+$, 447.1551, found 447.1539.

Prop-2-yn-1-yl 2-amino-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-6-(2-(trifluoromethyl)pyridin-4-yl)-4*H*-chromene-3-carboxylate (CXL145) (6i). White solid, yield 86%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.76 (d, *J* = 5.5 Hz, 1H), 7.84 (d, *J* = 1.0 Hz, 1H), 7.65 (dd, *J* = 5.0, 1.0 Hz, 1H), 7.61 (d, *J* = 2.0 Hz, 1H), 7.52 (dd, *J* = 8.5, 2.5 Hz, 1H), 7.13 (d, *J* = 8.5 Hz, 1H), 4.81-4.80 (m, 2H), 4.65-4.57 (m, 2H), 4.43 (dd, *J* = 7.5, 4.0 Hz, 1H), 2.77 (dd, *J* = 15.5, 4.0 Hz, 1H), 2.71 (dd, *J* = 15.5, 4.0 Hz, 1H), 2.48 (t, *J* = 2.5 Hz, 1H), 2.32 (t, *J* = 2.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.6, 167.8, 161.8, 158.2, 157.5, 149.2, 149.1, 148.8, 139.3, 137.5, 129.8, 126.4, 123.9, 122.7, 120.5,

118.2, 118.1, 78.5, 77.5,, 75.7, 74.8, 74.4, 51.9, 51.8, 51.3, 43.2, 30.9. HRMS (ESI+) m/z calcd for $C_{24}H_{18}F_3N_2O_5$ $[M+H]^+$, 471.1162, found 471.1149.

Prop-2-yn-1-yl 2-amino-6-(2,6-dimethylpyridin-4-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL143) (6j). Slightly yellow solid, yield 33% ; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.54 (d, *J* = 1.5 Hz, 1H), 7.45 (dd, *J* = 8.5, 2.0 Hz, 1H), 7.13 (s, 2H), 7.06 (d, *J* = 8.5 Hz, 1H), 4.82-4.74 (m, 2H), 4.61-4.56 (m, 2H), 4.41 (dd, *J* = 7.0, 4.5 Hz, 1H), 2.76 (dd, *J* = 15.5, 4.5 Hz, 1H), 2.70 (dd, *J* = 15.5, 7.5 Hz, 1H), 2.58 (s, 6H), 2.48 (t, *J* = 2.5 Hz, 1H), 2.31 (t, *J* = 2.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.6, 167.8, 162.1, 158.2, 150.4, 148.0, 135.2, 127.2, 126.6, 125.8, 118.1, 116.6, 78.6, 77.5, 75.8, 74.8, 74.4, 51.9, 51.2, 43.3, 31.1, 24.6. HRMS (ESI+) m/z calcd for $C_{25}H_{23}N_2O_5$ $[M+H]^+$, 431.1601, found 431.1587.

Prop-2-yn-1-yl 2-amino-6-(2-methoxypyridin-4-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL142) (6k). Slightly yellow solid, yield 67%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.19 (d, *J* = 5.5 Hz, 1H), 7.54 (d, *J* = 2.0 Hz, 1H), 7.45 (dd, *J* = 8.5, 2.5 Hz, 1H), 7.07-6.90 (m, 2H), 6.90 (s, 1H), 6.47 (brs, 2H), 4.80-4.77 (m, 2H), 4.61-4.42 (m, 2H), 4.42-4.39 (m, 1H), 3.98 (s, 3H), 2.78-2.67 (m, 2H), 2.47 (t, *J* = 2.5 Hz, 1H), 2.36 (t, *J* = 2.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 173.1, 167.4, 162.1, 159.7, 150.0, 151.8, 145.9, 137.4, 134.2, 127.3, 126.8, 125.9, 116.6, 105.3, 96.2, 78.7, 77.6, 75.9, 74.8, 55.7, 51.9, 51.4, 27.3, 24.5. HRMS (ESI+) m/z calcd for $C_{24}H_{21}N_2O_6$ $[M+H]^+$, 433.1394, found 433.1380.

Prop-2-yn-1-yl 2-amino-6-(2-(2-hydroxyethoxy)pyridin-4-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chro

mene-3-carboxylate (CXL139) (6l). Slightly yellow solid, yield 52%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.50 (d, *J* = 2.0 Hz, 1H), 7.42 (d, *J* = 2.5 Hz, 1H), 7.40 (d, *J* = 2.5 Hz, 1H), 7.05 (d, *J* = 8.5 Hz, 1H), 6.77 (s, 1H), 6.44 (dd, *J* = 7.0. 2.0 Hz, 1H), 6.42 (brs, 2H), 4.84-4.78 (m, 2H), 4.66-4.60 (m, 2H), 4.38 (dd, *J* = 7.5, 4.0 Hz, 1H), 4.17 (t, *J* = 4.0 Hz, 2H), 3.99 (t, *J* = 4.0 Hz, 2H), 2.73 (dd, *J* = 15.5, 9.0 Hz, 1H), 2.67 (dd, *J* = 15.5, 9.0 Hz, 1H), 2.48 (t, *J* = 2.5 Hz, 1H), 2.45 (t, *J* = 2.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.6, 167.7, 163.9, 161.9, 151.3, 150.9, 138.4, 133.6, 127.1, 126.5, 125.9, 116.7, 116.6, 105.8, 78.6, 77.6, 75.7, 75.1, 74.4, 62.1, 53.5, 51.9, 51.2, 43.2, 30.9. HRMS (ESI+) m/z calcd for $C_{25}H_{23}N_2O_7$ $[M+H]^+$, 463.1500, found 463.1486.

Prop-2-yn-1-yl

2-amino-6-(2-carbamoylpyridin-4-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL138) (6m). Slightly yellow solid, yield 71% ; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.60 (d, *J* = 5.0 Hz, 1H), 8.40 (s, 1H), 7.89 (s, 1H), 7.64-7.56 (m, 3H), 7.11 (d, *J* = 8.5Hz, 1H), 6.43 (brs, 2H), 5.62 (s, 1H), 4.81-4.79 (m, 2H), 4.65-4.56 (m, 2H), 4,41 (dd, *J* = 7.0, 4.5 Hz, 1H), 2.77 (dd, *J* = 15.5,3.5 Hz, 1H), 2.71 (dd, *J* = 15.5, 4.5 Hz, 1H), 2.48 (t, *J* = 2.0 Hz, 1H), 2.39 (t, *J* = 2.0 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.6, 166.7, 161.9, 159.7, 150.1, 149.1, 148.9, 148.8, 133.8, 127.3, 126.8, 126.2, 123.9, 120.1, 116.9, 78.6, 77.6, 75.8, 74.9, 74.4, 51.9, 51.3, 43.2, 31.0. HRMS (ESI+) m/z calcd for $C_{24}H_{20}N_3O_6$ $[M+H]^+$, 446.1347, found 446.1334.

Prop-2-yn-1-yl

2-amino-6-(2-((2-hydroxyethyl)carbamoyl)pyridin-4-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL135) (6n). Slightly yellow solid, yield 47%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.56 (d, *J* = 5.0 Hz, 1H), 8.37 (s, 1H), 7.62-7.55 (m, 3H)，7.09 (d, *J* = 8.5

Hz, 1H), 4.83-4.78 (m, 2H), 4.65-4.59 (m, 2H), 4.41(dd, *J* = 7.0, 4.5 Hz, 1H), 3.88 (t, *J* = 5.0 Hz, 1H), 3.68 (t, *J* = 5.0 Hz, 1H), 2.77 (dd, *J* = 15.5, 4.0 Hz, 1H), 2.71 (dd, *J* = 15.5, 7.5 Hz, 1H), 2.48 (s, 1H), 2.40 (s, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.6, 167.7, 165.5, 161.9, 150.9, 150.2, 148.9, 148.7, 133.8, 127.3, 126.8, 126.1, 123.7, 119.9, 116.9, 78.6, 77.6, 75.7, 75.0, 74.4, 62.6, 51.9, 51.3, 43.2, 42.7, 31.0. HRMS (ESI+) m/z calcd for $C_{26}H_{24}N_3O_7$ $[M+H]^+$, 490.1609, found 490.1595.

Prop-2-yn-1-yl 6-(2-acetamidopyridin-4-yl)-2-amino-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL140) (6o). White solid, yield 45%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.28 (s, 1H), 8.27 (s, 1H), 8.11 (s, 1H), 7.53 (dd, *J* = 8.5, 2.5 Hz, 1H), 7.58 (d, *J* = 2.0 Hz, 1H), 7.21 (dd, *J* = 5.5, 1.5 Hz, 1H), 7.08 (d, *J* = 8.0 Hz, 1H), 6.43 (brs, 2H), 4.83-4.76 (m, 2H), 4.65-4.57 (m, 2H), 4.40 (dd, *J* = 7.0, 4.0 Hz, 1H), 2.72 (dd, *J* = 16.0, 5.5 Hz, 1H), 2.48 (s, 1H), 2.23 (s, 3H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.6, 168.6, 167.8, 162.0, 152.0, 150.7, 149.9, 148.1, 127.3, 126.9, 125.8, 117.8, 111.4, 78.6, 77.6, 75.8, 74.8, 74.4, 51.9, 51.2, 43.1, 31.0, 24.9. HRMS (ESI+) m/z calcd for $C_{25}H_{22}N_3O_6$ $[M+H]^+$, 460.1503, found 460.1488.

Prop-2-yn-1-yl 6-(2-(N-acetylacetamido)pyridin-4-yl)-2-amino-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL141) (6p). White solid, yield 87%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.62 (d, *J* = 5.5 Hz, 1H), 7.59 (d, *J* = 2.0 Hz, 1H), 7.54 (dd, *J* = 5.5, 1.5 Hz, 1H), 7.51-7.49 (m, 1H), 7.41 (d, *J* = 1.0 Hz, 1H), 7.10 (d, *J* = 8.5 1H), 4.81-4.79 (m, 2H), 4.61-4.58 (m, 2H), 4.41 (dd, *J* = 7.0, 4.0 Hz, 1H), 2.77 (dd, *J* = 15.5, 4.5 Hz, 1H), 2.71 (dd, *J* = 15.5, 4.5 Hz, 1H), 2.49 (t, *J* = 2.0 Hz, 1H), 2.37 (t, *J* = 2.0 Hz, 1H), 2.32 (s, 6H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 172.6,

170.6, 167.7, 164.9, 153.4, 152.0, 150.6, 150.2, 143.8, 137.3, 129.4, 128.8, 126.3, 122.7, 121.5, 116.9, 78.5, 77.6, 75.7, 75.1, 74.4, 53.5, 51.9, 51.3, 43.2, 31.0, 26.7. HRMS (ESI+) m/z calcd for $C_{27}H_{24}N_3O_7$ $[M+H]^+$, 502.1609, found 502.1594.

Prop-2-yn-1-yl 2-amino-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-6-(2-propionamidopyridin-4-yl)-4*H*-chromene-3-carboxylate (CXL150) (6q). White solid, yield 71%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.50 (s, 1H), 8.48 (s, 1H), 8.27 (d, *J* = 5.0 Hz, 1H), 7.58 (d, *J* = 2.0 Hz, 1H), 7.54 (d, *J* = 8.5 Hz, 1H), 7.22 (dd, *J* = 5.0 Hz, 2.0 Hz, 1H), 7.06 (d, *J* = 8.5 Hz, 1H), 6.46 (brs, 1H), 4.81-4.79 (m, 2H), 4.65-4.41 (m, 2H), 4.40 (dd,2H, *J* = 6.5 Hz, 4.0 Hz, 1H), 2.74 (q, *J* = 7.5 Hz, 2H), 2.49-2.44 (m, 3H), 2.35 (t, *J* = 2.5 Hz, 1H), 1.27 (t, *J* = 7.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 172.5, 170.6, 167.8, 162.0, 152.2, 150.7, 149.8, 148.0, 134.6, 127.3, 126.7, 125.8, 117.6, 116.6, 111.5, 78.6, 77.6, 75.7, 74.8, 74.4, 51.9, 51.2, 43.1, 31.0, 30.8, 9.3. HRMS (ESI+) m/z calcd for $C_{26}H_{24}N_3O_6$ $[M+H]^+$, 474.1660, found 474.1645.

Prop-2-yn-1-yl 2-amino-6-(2-(2-hydroxyacetamido)pyridin-4-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL136) (6r). White solid, yield 79%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.08 (d, *J* = 5.0 Hz, 1H), 7.50 (d, *J* = 2.0 Hz, 1H), 7.42 (dd, *J* = 8.5, 2.0 Hz, 1H), 7.04 (d, *J* = 8.5 Hz, 1H), 6.83 (dd, *J* = 5.0, 1.5 Hz, 1H), 6.62 (s, 1H), 6.48 (brs, 2H), 4.80 (t, *J* = 2.0 Hz, 1H), 4.61-4.59 (m, 3H), 4.45-4.37 (m, 1H)), 2.77 (d, *J* = 4.0 Hz, 1H), 2.74 (d, *J* = 4.0 Hz, 1H), 2.49 (t, *J* = 2.5 Hz, 1H), 2.34 (t, *J* = 2.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.8, 168.5, 167.8, 162.2, 152.4, 150,8, 140.1, 148.2, 134.8, 127.6, 127.0, 125.5, 118.8, 117.6, 111.4, 78.5, 77.5, 75.9, 74.9, 74.5, 61.2, 51.9, 51.2, 43.1, 30.9. HRMS (ESI+) m/z calcd for $C_{25}H_{22}N_3O_7$ $[M+H]^+$, 476.1452, found 476.1451.

Prop-2-yn-1-yl 2-amino-6-(2-(methylamino)pyridin-4-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL134) (6s). White solid, yield 42%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.12 (d, *J* = 5.0 Hz, 1H), 7.52 (d, *J* = 2.0, 1H), 7.45 (dd, *J* = 8.0, 2.0 Hz, 1H), 7.25 (d, *J* = 8.5 Hz, 1H), 6.96 (dd, *J* = 5.0, 1.5 Hz, 1H), 6.51 (d, *J* = 1.5 Hz, 1H), 6.44 (brs, 2H), 4.80-4.79 (m, 2H), 4.64-4.59 (m, 2H), 4.41 (dd, *J* = 7.0, 4.0 Hz, 1H), 2.99 (d, *J* = 5.0 Hz, 3H), 2.76 (dd, *J* = 15.5, 4.5 Hz, 1H), 2.69 (dd, *J* = 15.0, 7.5 Hz, 1H), 2.47 (t, *J* = 2.5 Hz, 1H), 2.32 (t, *J* = 2.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.7, 167.8, 162.1, 160.1, 150.2, 149.0, 148.6, 145.8, 137.1, 126.6, 115.7, 111.4, 103.6, 78.6, 77.6, 75.9, 74.9, 74.3, 51.9, 51.2, 43.3, 31.1, 29.3. HRMS (ESI+) m/z calcd for $C_{24}H_{22}N_3O_5$ $[M+H]^+$, 432.1554, found 432.1541.

Prop-2-yn-1-yl 2-amino-6-(2-(ethylamino)pyridin-4-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL147）(6t). Slightly yellow solid, yield 58%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.09 (d, *J* = 5.0 Hz, 1H), 7.50 (d, *J* = 2.0 Hz, 1H), 7.42 (dd, *J* = 8.5 Hz, 2.0 Hz, 1H), 7.03 (d, *J* = 8.5 Hz, 1H), 6.73 (dd, *J* = 5.0 Hz, 1.5 Hz, 1H), 6.67 (brs, 2H), 6.50 (s, 1H), 4.79 (t, *J* = 2.0 Hz, 2H), 4.79-4.59 (m, 3H), 4.39 (dd, *J* = 7.0 Hz, 4.0 Hz, 1H), 3.36 (q, *J* = 7.0 Hz, 2H), 2.73 (dd, *J* = 10.5 Hz, 7.5 Hz, 1H), 2.68 (dd, *J* = 10.5 Hz, 7.5 Hz, 1H), 2.49 (t, *J* = 2.5 Hz, 1H), 2.34 (t, *J* = 2.5 Hz, 1H), 1.28 (t, *J* = 7.0 Hz, 3H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.7, 167.8, 162.2, 159.4, 150.2, 149.9, 148.6, 135.7, 127.0, 126.5, 125.7, 116.4, 111.2, 103.9, 78.7, 75.6, 75.0, 74.9, 74.4, 51.8, 51.2, 43.3, 37.0, 30.1, 14.9. HRMS (ESI+) m/z calcd for $C_{25}H_{24}N_3O_5$ $[M+H]^+$, 446.1710, found 446.1698.

Prop-2-yn-1-yl

2-amino-6-(2-(dimethylamino)pyridin-4-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL148) (6u). Slightly yellow solid, yield 71%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.20 (d, *J* = 5.0 Hz, 1H), 7.52 (d, *J* = 2.0 Hz, 1H), 7.44 (dd, *J* = 8.5 Hz, 2.0 Hz, 1H), 7.03 (d, *J* = 8.5 Hz, 1H), 6.71 (dd, *J* = 5.5 Hz, 1.5 Hz, 1H), 6.62 (s, 1H), 4.80-4.76 (m, 2H), 4.63-4.55 (m, 2H), 4.40 (dd, *J* = 7.5 Hz, 4.5 Hz, 1H), 3.14 (s, 6H), 2.75 (dd, *J* = 15.0 Hz, 4.5 Hz, 1H), 2.69 (dd, *J* = 15.5 Hz, 2.5 Hz, 1H), 2.48 (t, *J* = 2.0 Hz, 1H), 2.32 (t, *J* = 2.0 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.7, 167.8, 1 159.9, 150.1, 148.7, 148.3, 136.2, 127.1, 126.6, 116.4, 110.2, 77.6, 75.7, 74.9, 74.4, 51.8, 51.2, 43.3, 38.3, 31.3. HRMS (ESI+) m/z calcd for $C_{25}H_{24}N_3O_5$ $[M+H]^+$, 446.1710, found 446.1696.

Prop-2-yn-1-yl

2-amino-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-6-(2-(propylamino)pyridin-4-yl)-4*H*-chromene-3-carboxylate (CXL153) (6v). Slightly yellow solid, yield 71%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.10 (d, *J* = 5.0 Hz, 1H), 7.51 (d, *J* = 2.0 Hz, 1H), 7.04 (d, *J* = 8.5 Hz, 1H), 6.74 (dd, *J* = 5.0 Hz, 1.5 Hz, 1H), 6.49 (s, 1H), 6.48 (brs, 2H), 4.80-4.79 (m, 2H), 4.61-4.59 (m, 2H), 4.34 (dd, *J* = 7.5 Hz, 4.5 Hz, 1H), 3.30-3.27 (m, 2H), 2.72 (dd, *J* = 15.5 Hz, 4.5 Hz, 1H), 2.67 (dd, *J* = 15.5 Hz, 7.5 Hz, 1H), 2.48 (t, *J* = 2.5 Hz, 1H), 2.32 (t, *J* = 2.5 Hz, 1H), 1.71-1.66 (m, 2H), 1.03 (t, *J* = 7.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.7, 167.8, 162.1, 159.5, 150.2, 148.9, 148.7, 135.8, 127.0, 126.5, 125.7, 116.4, 111.2, 103.8, 78.6, 77.6, 75.8, 74.9, 74.3, 51.8, 51.2, 44.2, 43.3, 31.1, 22.8, 11.6. HRMS (ESI+) m/z calcd for $C_{26}H_{26}N_3O_5$ $[M+H]^+$, 460.1867, found 460.1852.

Prop-2-yn-1-yl

2-amino-6-(2-(N-methylacetamido)pyridin-4-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-c

hromene-3-carboxylate (CXL151) (6w). Slightly yellow solid, yield 62%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.50 (d, *J* = 5.5 Hz, 1H), 7.58 (d, *J* = 2.0 Hz, 1H), 7.53-7.46 (m, 2H), 7.36 (d, *J* = 4.0 Hz, 1H), 7.10 (d, *J* = 8.5 Hz, 1H), 6.52 (brs, 2H), 4.81-4.79 (m, 2H), 4.61-4.59 (m, 2H), 4.41 (dd, *J* = 7.5 Hz, 4.0 Hz, 1H), 3.44 (s, 3H), 2.77 (dd, *J* = 15.5 Hz, 4.5 Hz, 1H), 2.71 (dd, *J* = 15.5, 7.5 Hz, 1H), 2.49 (t, *J* = 2.5 Hz, 1H), 2.35 (t, *J* = 2.5 Hz, 1H), 2.17 (d, *J* = 6.0 Hz, 3H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.8, 170.6, 167.7, 161.2, 150.9, 150.1, 149.2, 133.9, 127.4, 126.7, 126.2, 119.6, 118.0, 116.9, 116.1, 78.6, 77.6, 75.7, 74.9, 74.1, 51.9, 51.3, 43.2, 35.9, 30.9, 23.2. HRMS (ESI+) m/z calcd for $C_{26}H_{24}N_3O_6$ $[M+H]^+$, 474.1660, found 474.1645.

Prop-2-yn-1-yl

2-amino-6-(6-aminopyridin-3-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-car

boxylate (CXL155) (6x). Slightly yellow solid, yield 43%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.25 (d, *J* = 1.5 Hz, 1H), 7.59 (dd, *J* = 8.5 Hz, 1.5 Hz, 1H), 7.38 (s, 1H), 7.31 (d, *J* = 8.5 Hz, 1H), 7.00 (d, *J* = 3.0 Hz, 1H), 6.55 (d, *J* = 8.5 Hz, 1H), 6.53 (brs, 2H), 4.83-4.76 (m, 2H), 4,64 (s, 2H), 4.60-4.56 (m, 2H), 4.39 (dd, *J* = 7.5 Hz, 4.0 Hz, 1H), 2.74 (dd, *J* = 15.5 Hz, 8.0 Hz, 1H), 2.68 (dd, *J* = 15.5 Hz, 8.0 Hz, 1H), 2.49 (s, 1H), 2.36 (s, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.7, 167.9, 162.3, 157.7, 149.0, 146.1, 136.4, 134.9, 126.1, 125.7, 125.6, 116.4, 77.6, 75.7, 75.0, 74.4, 51.8, 51.1, 43.4, 31.1. HRMS (ESI+) m/z calcd for $C_{23}H_{20}FN_3O_5$ $[M+H]^+$, 418.13971, found 418.1401.

Prop-2-yn-1-yl

2-amino-6-(6-methylpyridin-3-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-car

boxylate (CXL161) (6y). Slightly yellow solid, yield 49%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.68 (d, *J* = 2.0 Hz, 1H), 7.71-7.65 (m, 2H), 7.48 (s, 1H), 7.39 (d, *J* = 2.5 Hz, 1H), 7.21 (d, *J* = 8.0 Hz,

1H), 7.07 (d, *J* = 8.5 Hz, 1H), 6.45 (brs, 2H), 4.80-4.79 (m, 2H), 4.61-4.59 (m, 2H), 4.41 (dd, *J* = 7.5 Hz, 4.5 Hz, 1H), 2.70 (dd, *J* = 15.5 Hz, 7.5 Hz, 1H), 2.60 (s, 3H), 2.47 (t, *J* = 2.5 Hz, 1H), 2.32 (t, *J* = 2.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.7, 167.8, 162.2, 147.3, 134.5, 132.8, 132.0, 128.7, 127.0, 126.5, 125.9, 123.2, 78.6, 77.6, 75.8, 74.9, 74.3, 51.8, 51.2, 43.3, 31.1, 24.1. HRMS (ESI+) m/z calcd for $C_{24}H_{21}N_2O_5$ $[M+H]^+$, 417.1445, found 417.1445.

Prop-2-yn-1-yl

2-amino-6-(6-fluoropyridin-3-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carb oxylate (CXL162) (6z). Slightly yellow solid, yield 64%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.37 (d, *J* = 2.5 Hz, 1H), 7.91 (dd, *J* = 8.5 Hz, 2.0 Hz, 1H), 7.46 (d, *J* = 2.0 Hz, 1H), 7.37 (dd, *J* = 8.5 Hz, 2.0 Hz, 1H), 7.08 (d, *J* = 8.5 Hz, 1H), 6.99 (dd, *J* = 8.5 Hz, 3.0 Hz, 1H), 4.80-4.79 (m, 2H), 4.62-4.60 (m, 2H), 4.41 (dd, *J* = 7.5 Hz, 4.0 Hz, 1H), 2.77 (dd, *J* = 15.5 Hz, 4.0 Hz, 1H), 2.69 (dd, *J* = 15.5 Hz, 7.5 Hz, 1H), 2.48 (t, *J* = 2.5 Hz, 1H), 2.35 (t, *J* = 2.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.7, 167.8, 164.0, 162.1, 162.0, 149.9, 145.7, 145.6, 139.6, 133.9, 133.2, 127.2, 126.6, 126.1, 109.6, 109.3, 78.6, 77.6, 75.8, 75.7, 74.9, 74.4, 65.9, 52.8, 51.9, 51.2, 43.3, 31.0, 29.7. HRMS (ESI+) m/z calcd for $C_{23}H_{18}FN_2O_5$ $[M+H]^+$, 421.1194, found 421.1192.

Prop-2-yn-1-yl

2-amino-6-(5-fluoropyridin-3-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carb oxylate (CXL157) (6aa). Slightly yellow solid, yield 79%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.63 (s, 1H), 8.44 (d, *J* = 2.5 Hz, 1H), 7.57-7.51 (m, 2H), 7.09 (d, *J* = 8.5 Hz, 1H), 6.49 (brs, 2H), 4.81-4.79 (m, 2H), 4.63-4.61 (m, 2H), 4.42 (dd, *J* = 8.0 Hz, 4.0 Hz, 1H), 2.76 (dd, *J* = 15.5 Hz, 4.0 Hz, 1H), 2.70 (dd, *J* = 15.5 Hz, 7.5 Hz, 1H), 2.49 (t, *J* = 2.0 Hz, 1H), 2.36 (t, J = 2.0 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.6, 67.8, 162.0, 160.7, 158.7, 150.9, 148.8, 146.9, 137.8, 136.7,

136.2, 132.9, 127.5, 126.8, 120.9, 120.8, 77.7, 75.8, 74.3, 74.0, 51.9, 51.4, 43.3, 30.9. HRMS (ESI+) m/z calcd for $C_{23}H_{18}F_2N_3O_5$ $[M+H]^+$, 421.1994, found 421.1993.

Prop-2-yn-1-yl 2-amino-6-(5-methylpyridin-3-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL158) (6ab). Slightly yellow solid, yield 65%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.60 (d, *J* = 2.0 Hz, 1H), 8.41 (s, 1H), 7.62 (s, 1H), 7.48 (d, *J* = 2.0 Hz, 1H), 7.07 (d, *J* = 8.5 Hz, 1H), 4.82-4.79 (m, 2H), 4.65-4.56 (m, 2H), 4.41 (dd, *J* = 7.5 Hz, 4.0 Hz, 1H), 2.76 (dd, *J* = 15.5 Hz, 4.5.0 Hz, 1H), 2.70 (dd, *J* = 15.0 Hz, 7.5 Hz, 1H), 2.48 (t, *J* = 2.5 Hz, 1H), 2.40 (s, 3H), 2.31 (t, *J* = 2.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.7, 167.8, 162.2, 149.8, 148.6, 145.5, 134.8, 133.4, 127.9, 126.7, 125.6, 78.7, 77.9, 75.8, 74.9, 74.4, 51.8, 51.2, 43.3, 31.1, 30.9. HRMS (ESI+) m/z calcd for $C_{24}H_{21}N_2O_5$ $[M+H]^+$, 417.1445, found 417.1452.

Prop-2-yn-1-yl 2-amino-6-(5-methoxypyridin-3-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL159) (6ac). Slightly yellow solid, yield 69%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.41 (s, 1H), 8.28 (d, *J* = 2.5 Hz, 1H), 7.49 (d, *J* = 2.5 Hz, 1H), 7.32 (dd, *J* = 7.5 Hz, 2.0 Hz, 1H), 7.08 (d, *J* = 8.5 Hz, 1H), 4.80-4.79 (m, 2H), 4.62-4.56 (m, 2H), 4.42 (dd, *J* = 7.5 Hz, 4.5 Hz, 1H), 3.93 (s, 3H), 2.76 (dd, *J* = 15.5 Hz, 4.0 Hz, 1H), 2.69 (dd, *J* = 15.0 Hz, 7.5 Hz, 1H), 2.48 (t, *J* = 2.5 Hz, 1H), 2.33 (t, *J* = 2.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.6, 167.8, 162.0, 156.7, 150.0, 140.8, 136.4, 136.1, 134.2, 126.8, 125.9, 114.0, 116.6, 78.5, 77.6, 75.9, 74.8, 74.2, 55.7, 51.9, 51.2, 43.3, 31.0. HRMS (ESI+) m/z calcd for $C_{24}H_{21}N_2O_6$ $[M+H]^+$, 433.1394, found 433.1396.

Prop-2-yn-1-yl 2-amino-6-(5-aminopyridin-3-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL160) (6ad). Slightly yellow solid, yield 54%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.19 (s, 1H), 8.06 (d, *J* = 2.0 Hz, 1H), 7.44 (s, 1H), 7.10 (s, 1H), 7.02 (d, *J* = 8.0 Hz, 1H), 6.54 (brs, 2H), 4.83-4.76 (m, 2H), 4.64-4.59 (m, 2H), 4.40 (dd, *J* = 7.5 Hz, 4.5 Hz, 1H), 3.78 (s, 2H), 2.76 (dd, *J* = 15.5 Hz, 4.0 Hz, 1H), 2.68 (dd, *J* = 15.5 Hz, 7.5 Hz, 1H), 2.47 (s, 1H), 2.34 (s, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.7, 167.9, 162.9, 149.8, 142.4, 138.6, 136.3, 136.0, 139.9, 134.7, 126.7, 125.8, 119.8, 116.5, 78.7, 77.6, 75.9, 74.9, 74.3, 51.9, 51.3, 43.4, 31.1. HRMS (ESI+) m/z calcd for $C_{23}H_{20}N_3O_5$ $[M+H]^+$, 418.1397, found 418.1395.

Prop-2-yn-1-yl 6-(6-acetamidopyridin-3-yl)-2-amino-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL163) (6ae). Slightly yellow solid, yield 71%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.54-8.51 (m, 2H), 8.32 (s, 1H), 7.49 (d, *J* = 2.0 Hz, 1H), 7.43 (dd, *J* = 8.5 Hz, 2.5 Hz, 1H), 7.07 (d, *J* = 8.5 Hz, 1H), 6.39 (brs, 2H), 4.80-4.79 (m, 2H), 4.65-4.57 (m, 2H), 4.40 (dd, *J* = 7.0 Hz, 4.5 Hz, 1H), 2.76 (dd, *J* = 15.5 Hz, 4.5 Hz, 1H), 2.70 (dd, *J* = 15.0 Hz, 7.0 Hz, 1H), 2.48 (t, *J* = 2.5 Hz, 1H), 2.35 (t, *J* = 2.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.7, 168.7, 167.8, 162.1, 150.1, 143.7, 139.7, 136.0, 134.7, 133.9, 127.3, 126.9, 125.9, 125.3, 116.7, 78.6, 77.6, 75.8, 74.9, 74.4, 51.9, 51.2, 43.3, 31.0, 24.6. HRMS (ESI+) m/z calcd for $C_{25}H_{22}N_3O_6$ $[M+H]^+$, 460.1503, found 460.1505.

Prop-2-yn-1-yl 2-amino-6-(1-methyl-1H-pyrazol-4-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL152) (6af). Slightly yellow solid, yield 54%; ^{1}H-NMR ($CDCl_3$, 500 MHz):

δ 7.70 (s, 1H), 7.56 (s, 1H), 7.35 (d, *J* = 2.0 Hz, 1H), 7.28 (dd, *J* = 8.5 Hz, 2.0 Hz, 1H), 6.96 (d, *J* = 8.5 Hz, 1H), 4.80-4.79 (m, 2H), 4.65-4.56 (m, 2H), 4.34 (dd, *J* = 7.5 Hz, 4.5 Hz, 1H), 3.93 (s, 3H), 2.74 (dd, *J* = 15.0 Hz, 4.5 Hz, 1H), 2.68 (dd, *J* = 15.5 Hz, 7.5 Hz, 1H), 2.47 (t, *J* = 2.5 Hz, 1H), 2.37 (t, *J* = 2.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.7, 167.9, 148.2, 136.7, 129.3, 126.8, 125.6, 125.3, 125.0, 122.4, 77.7, 75.8, 74.9, 74.2, 51.8, 51.1, 43.3, 39.1, 31.0. HRMS (ESI+) m/z calcd for $C_{22}H_{20}N_3O_5$ $[M+H]^+$, 406.1397, found 406.1384.

Prop-2-yn-1-yl 2-amino-6-(1-methyl-1H-pyrazol-3-yl)-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (CXL154) (6ag). Slightly yellow solid, yield 52%; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.66-7.63 (m, 2H), 7.36 (d, *J* = 2.0 Hz, 1H), 6.99 (dd, *J* = 8.0 Hz, 1H), 6.48 (d, *J* = 2.5 Hz, 1H), 6.41 (brs, 2H), 4.81-4.75 (m, 2H), 4.63-4.54 (m, 2H), 4.36 (t, *J* = 5.5 Hz, 1H), 3.94 (s, 3H), 2.75 (d, *J* = 5.5 Hz, 1H), 2.46 (t, *J* = 2.5 Hz, 1H), 2.37 (t, *J* = 2.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.7, 168.0, 162.3, 150.7, 149.4, 131.4, 130.4, 125.4, 125.1, 116.1, 102.7, 78.7, 77.7, 75.7, 74.8, 74.2, 51.8, 51.1, 43.1, 39.0, 31.1. HRMS (ESI+) m/z calcd for $C_{22}H_{20}N_3O_5$ $[M+H]^+$, 406.1397, found 406.1382.

Prop-2-yn-1-yl 2-amino-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-6-(tetrazolo[1,5-a]pyridin-7-yl)-4*H*-chromene-3-carboxylate (CXL156) (6ah). ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.89 (d, *J* = 8.5 Hz, 1H), 8.13 (s, 1H), 7.67 (d, *J* = 2.0 Hz, 1H), 7.15 (d, *J* = 8.5 Hz, 1H), 6.56 (brs, 2H), 4.85-4.78 (m, 2H), 4.68-4.59 (m, 2H), 4.44 (dd, *J* = 7.5 Hz, 4.0 Hz, 1H), 2.80 (dd, *J* = 15.5 Hz, 8.0 Hz, 1H), 2.72 (dd, *J* = 15.5 Hz, 8.0 Hz, 1H), 2.51 (t, *J* = 2.0 Hz, 1H), 2.46 (t, *J* = 2.0 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 125 MHz): 170.7, 167.6, 161.9, 151.1, 149.2, 144.3, 133.0, 127.7, 126.9, 126.5, 125.2,

117.2, 116.7, 77.6, 75.6, 75.1, 74.5, 51.9, 51.3 43.2, 30.9. HRMS (ESI+) m/z calcd for $C_{23}H_{18}N_5O_5$ $[M+H]^+$, 444.1302, found 444.1302.

Cell Culture. CCl-240 (HL-60) and CRL-2257 (HL-60/MX-2) were purchased from ATCC. OVCAR-8 and NCI/ADR-RES were purchased from NCI 60 cancer cell line panel. All of these cell lines were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS). Medium was changed every 2 days. Cells were cultured at 37□ with 5% CO_2 in air atmosphere.

Cytosolic Ca^{2+} measurement from ER. Fluo-4AM Direct™ Calcium assay kits (Molecular Probe) was used to measure the cytosolic Ca^{2+} concentration following the procedures provided. Briefly, HL-60 or HL-60/MX-2 cells were centrifuged at 250g for 5 min to remove medium and resuspended in HEPES and HBSS buffer containing 1.3 mM $CaCl_2$ at a cell density of 5 million/mL. Same volume of Fluo-4AM solution with 2.5 mM probenecid was mixed with cells and incubated for 30 min at 37□. 100 μL cells were transferred to each well of a 96-well Costar black plate. To each well was added 50 μL compound solution of varying concentrations in HEPES and HBSS buffer containing 2.8 mM EGTA with 1% DMSO. The fluorescence signal (excitation 485 nm and emission 525 nm) was measured by a microplate reader (Synergy H1, BioTek). For OVCAR-8 and NCI/ADR-RES, 5000 cells in 100 μL medium were seeded in each well of clear 96-well plates. After incubation at 37°C overnight, medium was removed and 50 μL HEPES and HBSS buffer and 50 μL Fluo-4 AM solution with 2.5 mM probenecid were added to each well. Cells were incubated for 30 min at 37°C. Thapsigargin (200 nM) was used to empty ER Ca^{2+} and the fluorescence signal increase induced by ER Ca^{2+} releasing was monitored to

quantify ER calcium content. The cytosolic concentration of Ca^{2+} induced by compounds was quantified according to the following formula:[40]

$$[Ca^{2+}] = K_d[(F - F_{min})/(F_{max} - F)]$$

where K_d is the dissociation constant of the fluorescent dye with Ca^{2+}, F is the fluorescence intensity, F_{min} is the fluorescence intensity in the absence of Ca^{2+} treated with EGTA and ionomycin while F_{max} is the fluorescence intensity with saturating Ca^{2+} upon ionomycin treatment.

Evaluation of Cell Viability. The *in vitro* anti-proliferative activity of these compounds was assayed by determining their ability to inhibit cell growth. Cells were seeded in a clear 96-well plat at 10^4 cells/well. Compounds were added at varying concentrations with 1% DMSO. After 48 hr treatment, relative cell viability was measured using CellTiter-Blue Cell Viability Assay. The GI_{50} of each compound was determined by fitting the cell relative viability to compound concentration using GraphPad. For BAPTA protection, cells were pre-treated with BAPTA ester for one hour followed by CXL compound treatment for 48 hours.

Statistical analysis Data were reported as mean ± standard deviation (SD). The student two-tailed *t* test was used for comparisons of the data between different groups, such as HL60 and HL60/MX2. P value ≤ 0.05 was considered statistically significant. All analyses were conducted in GraphPad Prism 4 (GraphPad Software, Inc.).

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Supporting information Availability The Supporting Information is available free of charge on the ACS Publication website at http://pubs.acs.org.

List of Supporting Information:

1. Synthetic procedures of CXL compounds.
2. Characterizations of the intermediates.
3. Molecular Formular Strings

Corresponding Author Information

Corresponding Authors:

Chengguo Xing: chengguoxing@cop.ufl.edu

Haifeng Sun: sunhaifengliu@163.com

Abbreviations

Multi-drug resistance (MDR), acute myeloid leukemia (AML), structure activity relationship (SAR), mitoxantrone (MX), ATP-binding cassette (ABC), endoplasmic reticulum (ER), sarco endoplasmic reticulum ATPase (SERCA), inositol triphosphate receptor (IP_3R), 50% growth inhibitory concentration (GI_{50}), National Cancer Institute (NCI), cytarabine (Ara-C).

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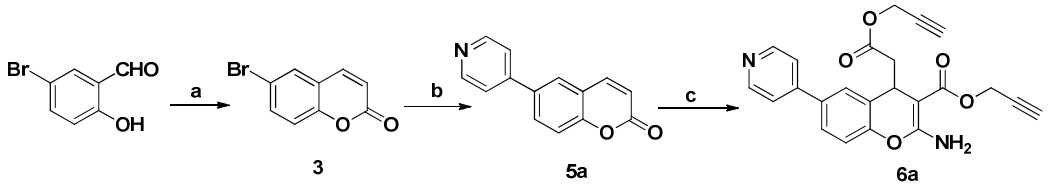
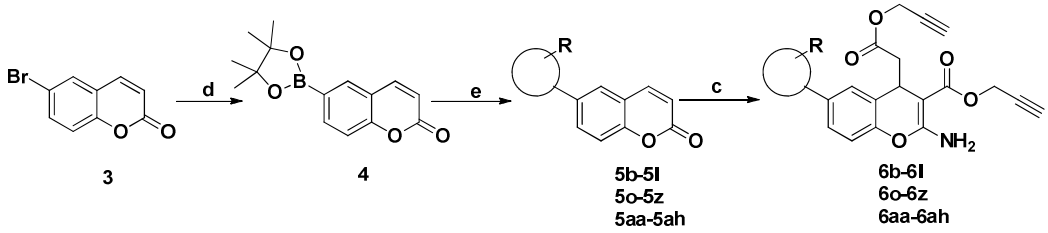
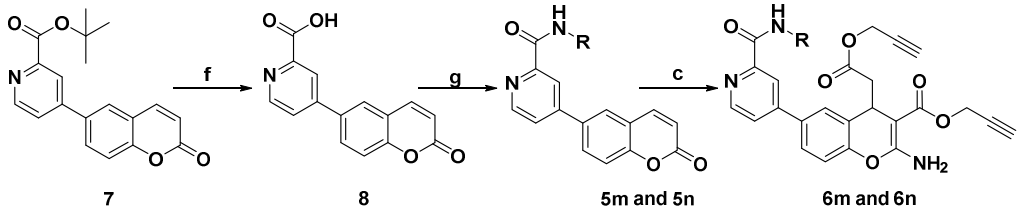
Scheme 1. Syntheses of chromene-based CXL series of analogs.



Reagents and conditions: **a**, DMA, $POCl_3$, DCM, reflux; **b**, 4-pyridine boronic acid, $Pd(PPh_3)_4$, EtOH/toluene, K_2CO_3, heat; **c**, ethyl cyanoacetate, propargyl alcohol, Na.



Reagents and conditions: **d**, $Pd(dppf)Cl_2$, DME, KOAc, Bis(pinocolato)diboron, reflux; **e**, $Pd(PPh_3)_4$, DME, 2N Na_2CO_3 aqueous, reflux.



Reagents and conditions: **f**, TFA, DCM, room temperature; **g**, EDCI, HOBt, DIPEA, DCM, room temperature.

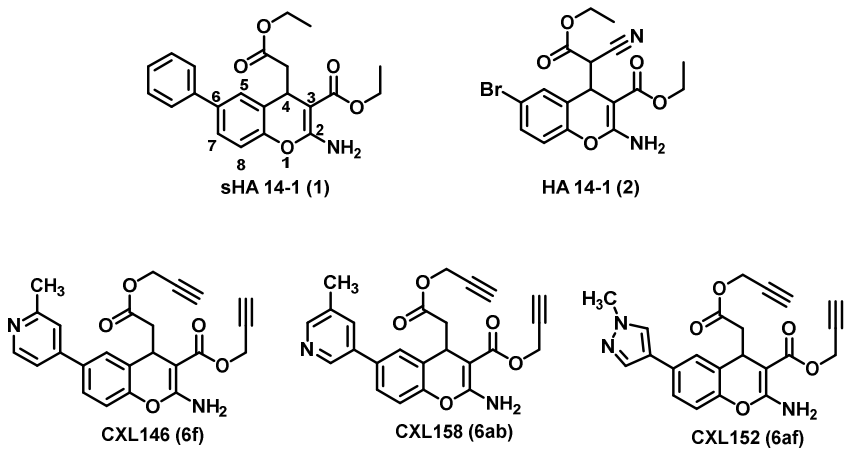


Figure 1. Structures of sHA 14-1, HA 14-1 and three lead CXL candidates.

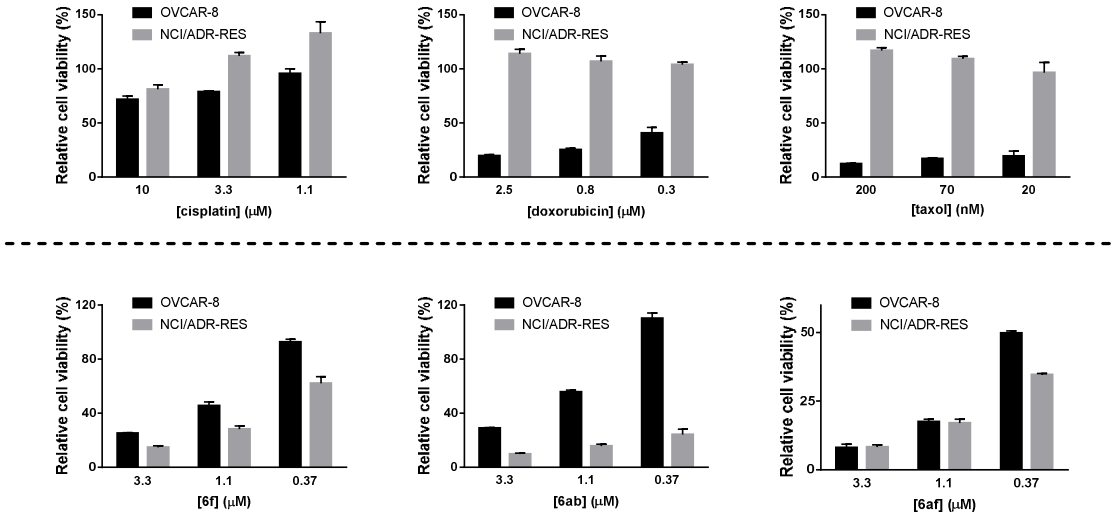


Figure 2. Relative sensitivity of OVCAR-8 ovarian cancer cell line and the MDR NCI/ADR-RES cell line toward three conventional chemotherapies and three CXL compounds (n = 3).

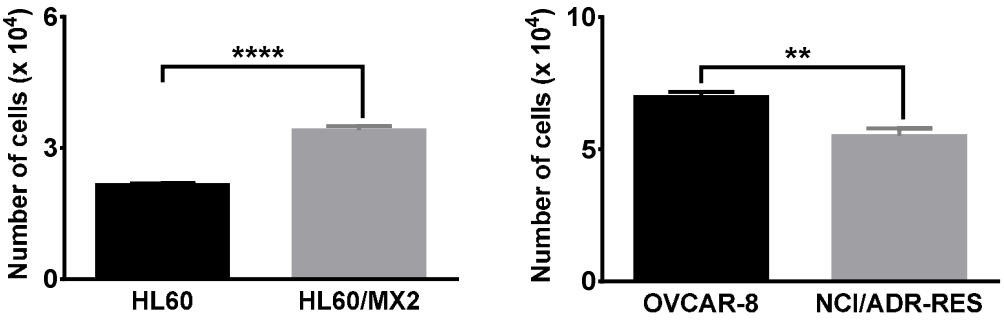


Figure 3. Forty-eight hour cell proliferation between parental and MDR cancer cell lines (n = 6).

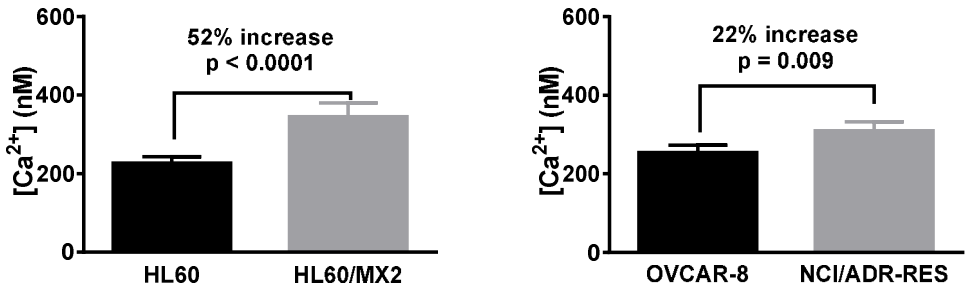


Figure 4. ER calcium contents in HL60, HL60/MX2 and OVCAR-8, NCI/ADR-RES cell lines (n = 4 – 6).

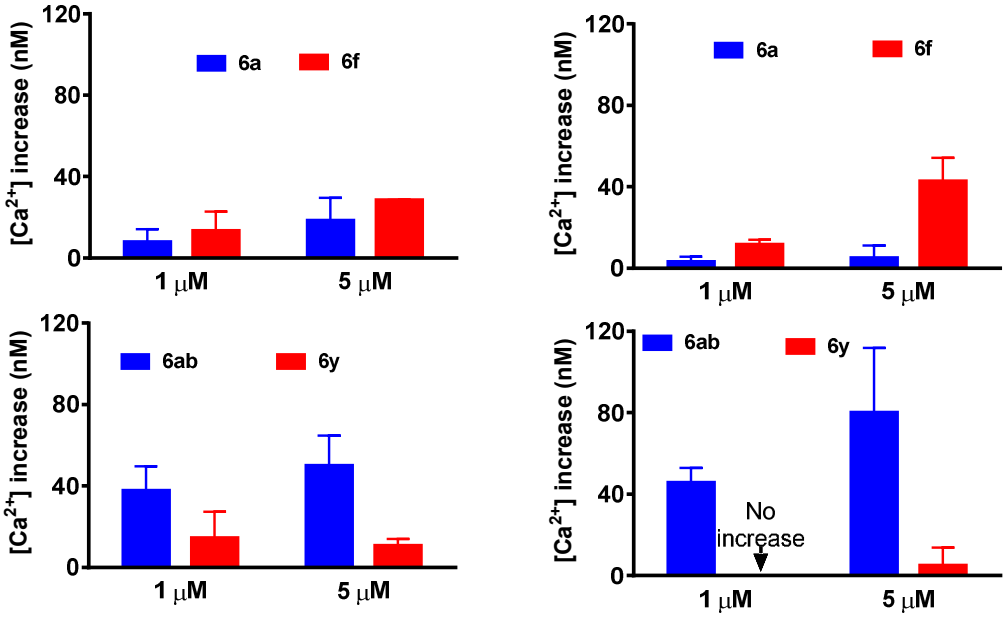


Figure 5. The increase in cytosolic calcium upon **6a**, **6f**, **6ab** and **6y** treatment in HL60 (left) and HL60/MX2 (right) (n = 3 – 4).

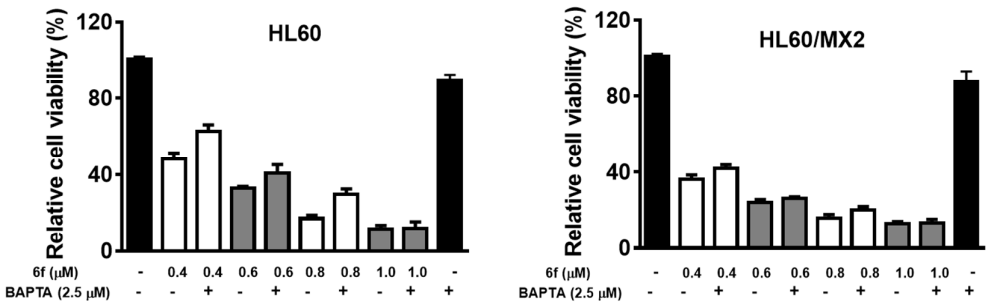
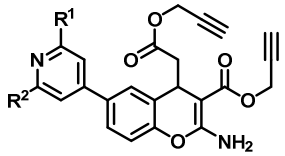


Figure 6. The effect of chelating intracellular calcium on the anti-proliferative activity of CXL146 in HL60 and HL60/MX2 cells.

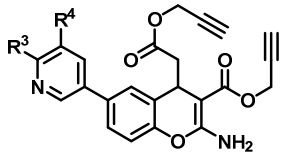
Table 1. The anti-proliferative activities of CXL compounds with substituents at the 3′-position of pyridine against HL60 and HL60/MX2 cell lines.



No.	R^1	R^2	GI_{50} (μM)		Selectivity
			HL60/MX2	HL60	HL60/(HL60/MX2)
CXL131 (6a)	H	H	12.6±1.7	25.6±7.1	2.0
CXL132 (6b)	H	$-NH_2$	2.7±0.9	7.9±1.8	3.0
CXL133 (6c)	H	-OH	4.2±0.3	14.1±1.2	3.4
CXL164 (6d)	H	-F	7.1±0.7	28.6±2.0	4.1
CXL144 (6e)	H	-CN	22.7±1.7	81.4±8.0	3.6
CXL146 (6f)	H	$-CH_3$	0.17±0.03	0.48±0.07	2.8
CXL137 (6g)	H	$-CH_2OH$	0.73±0.2	1.8±0.2	2.5
CXL149 (6h)	H	$-CH_2OCH_3$	6.6±0.2	11.0±3.8	1.7
CXL145 (6i)	H	$-CF_3$	0.92±0.03	3.3±0.9	3.6
CXL143 (6j)	CH_3	$-CH_3$	1.5±0.3	3.9±0.2	3.1
CXL142 (6k)	H	$-OCH_3$	2.1±0.6	4.5±0.8	2.2
CXL139 (6l)	H	$-OCH_2CH_2OH$	31.7±3.6	31.2±1.1	1.0
CXL138 (6m)	H	$-CONH_2$	1.7±0.2	4.3±0.9	3.0
CXL135 (6n)	H	$-CONHCH_2CH_2OH$	12.1±2.6	11.1±2.6	0.9
CXL140 (6o)	H	$-NHCOCH_3$	0.59±0.09	1.2±0.1	2.0
CXL141 (6p)	H	$-N(COCH_3)_2$	0.60±0.03	1.7±0.1	2.8
CXL150 (6q)	H	$-NHCOCH_2CH_3$	0.94±0.16	1.07±0.04	1.1
CXL136 (6r)	H	$-NHCOCH_2OH$	1.36±0.06	1.52±0.04	1.1
CXL134 (6s)	H	$-NHCH_3$	0.39±0.04	0.88±0.07	2.3
CXL147 (6t)	H	$-NHCH_2CH_3$	1.18±0.36	1.81±0.54	1.5
CXL148 (6u)	H	$-N(CH_3)_2$	0.39±0.09	0.81±0.03	2.1
CXL153 (6v)	H	$--NHCH_2CH_2CH_3$	11.1±0.7	9.8±2.8	0.9
CXL151 (6w)	H	$-NCH_3COCH_3$	1.69±0.04	3.3±0.1	1.9

Results are given as the mean of at least two independent experiments with triplicates in each experiment. Shaded are the compounds of poor selectivity.

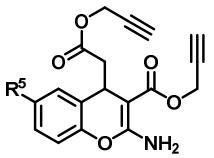
Table 2. The anti-proliferative activities of compounds with substituents at the 2' or 3'-position of pyridine against HL60 and HL60/MX2 cell lines.

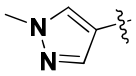
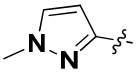
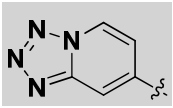


No.	R^3	R^4	GI_{50} (μM)[a]		Selectivity
			HL60/MX2	HL60	HL60/(HL60/MX2)
CXL155 (6x)	$-NH_2$	H	1.4±0.4	5.3±1.6	3.8
CXL161 (6y)	$-CH_3$	H	7.8±0.8	21.9±3.6	2.8
CXL162 (6z)	-F	H	1.8±0.3	12.1±0.9	6.8
CXL157 (6aa)	H	-F	1.0±0.2	3.8±0.2	3.8
CXL158 (6ab)	H	$-CH_3$	0.19±0.06	0.38±0.03	2.0
CXL159 (6ac)	H	$-OCH_3$	0.9±0.1	5.3±0.2	5.9
CXL160 (6ad)	H	$-NH_2$	0.47±0.09	1.3±0.4	2.7
CXL163 (6ae)	H	$-NHCOCH_3$	16.0±4.2	1.7±0.1	0.1

[a]Results are given as the mean of at least two independent experiments with triplicates in each experiment. Shaded are the compounds of poor selectivity.

Table 3. The anti-proliferative activities of compounds with heterocyclic substituents against HL60 and HL60/MX2 cell lines.



No.	R^5	GI_{50} (μM)		Selectivity
		HL60/MX2	HL60	HL60/(HL60/MX2)
CXL152 (6af)		0.099±0.025	0.26±0.04	2.7
CXL154 (6ag)		1.63±0.05	8.9±1.2	5.5
CXL156 (6ah)		43.9±5.8	12.17±6.38	0.3

Results are given as the mean of at least two independent experiments with triplicates in each experiment. Shaded are the compounds of poor selectivity.

Table 4. GI_{50} values of three lead compounds and two standard therapies on cord blood and bone marrow cells in comparison to HL60 and HL60/MX2 cells.

No.	GI_{50} (μM)			
	Cord blood cells	Bone marrow cells	HL60	HL60/MX2
CXL146 (**6f**)	>100	>100	0.48	0.17
CXL152 (**6ab**)	>100	>100	0.38	0.19
CXL158 (**6af**)	>100	>100	0.26	0.099
Cytarabine	>100	64.1	1.0	1.8
Mitoxantrone	62.9	7.0	0.019	0.54

Cord blood cells were from two different donors and bone marrow cells was from one donor.

Image Manipulation

ChemDraw Professional

GraphPad Prism

Powerpoint

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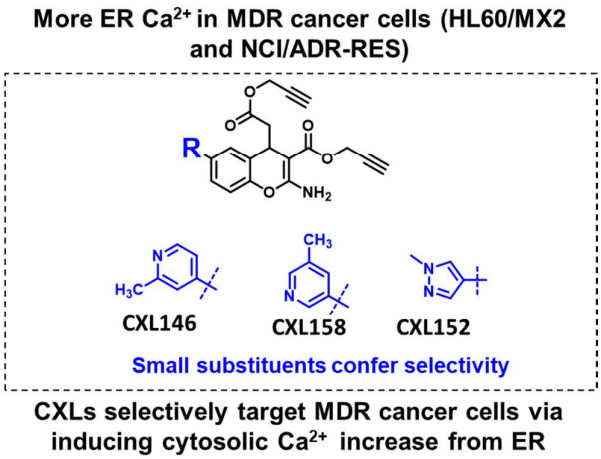

Molecular Formula Strings: attached.