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4*H*-Chromene-based anticancer agents towards multi-drug resistant HL60/MX2 human leukemia: SAR at the 4th and 6th positions

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Abstract

4*H*-Chromene-based compounds, for example, CXL017, CXL035, and CXL055, have a unique anticancer potential that they selectively kill multi-drug resistant cancer cells. Reported herein is the extended structure–activity relationship (SAR) study, focusing on the ester functional group at the 4th position and the conformation at the 6th position. Sharp SARs were observed at both positions with respect to cellular cytotoxic potency and selectivity between the parental HL60 and the multi-drug resistant HL60/MX2 cells. These results provide critical guidance for future medicinal optimization.

Keywords

4*H*-Chromene; Anticancer; Cytotoxicity; Multi-drug resistant; Structure–activity relationship

1. Introduction

Over 500,000 people would die of cancer each year in the U.S.A. alone.^{1,2} One major challenge associated with the limited success of cancer treatment is drug resistance.³ Cancers, irrespective of origins, can be intrinsically resistant to therapies or acquire resistance during treatment, rendering cancer therapies less effective and ultimately leading to death among cancer patients. For instance, the typical response period of a cancer patient to kinase-based inhibitors is only 6–30 months.⁴ Therefore, novel therapies are urgently needed that can effectively target multiple drug-resistant malignancies, which may significantly improve the outcome of cancer treatment.

At the molecular level, cancer cells can acquire drug resistance via different mechanisms because of the cellular heterogeneity and evolving nature. Typical mechanisms include

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alterations in drug transport, mutations in target proteins, changes in cellular repair machineries, or compromised cellular sensitivity to death signals caused by cancer therapies.^{8–10} It is ideal to identify chemical templates that can simultaneously tackle drug resistance acquired through varied mechanisms. Our earlier work discovered a series of 4*H*-chromene-based compounds (representatives in Fig. 1), which preferentially kill several multi-drug resistant cancer cells even though these cancer cells gain drug resistance via different mechanisms.^{5,6} For instance, CXL055 (**3**) demonstrates equal or better cytotoxicity against nine multi-drug resistant cancer cell lines. More importantly, unlike standard cancer therapies, including chemotherapies and targeted therapies, cancer cells failed to acquire resistance to the 4*H*-chromene-based compound, for example, CXL017 (**1**), even upon chronic exposure.¹¹ Indeed, HL60/MX2, a multi-drug resistant human leukemia cell line, became re-sensitized to standard therapies upon chronic exposure to **1**, substantiating the unique anticancer potential of these 4*H*-chromene-based compounds towards drug-resistant cancers. Mechanistically these lead candidates inhibit sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) via a unique binding interaction¹² and modulate SERCA expression in cells.¹¹ SERCA, although previously regarded as undruggable for cancer treatment, was recently demonstrated to be a promising target, particularly towards multi-drug resistant malignancies.^{3,13} 4*H*-Chromene-based anti-cancer agents therefore may be complementary to current cancer therapies for successful cancer management.

Our earlier structure–activity relationship (SAR) studies of the 4*H*-chromene-based compounds have revealed sharp sensitivity of their cytotoxic potency to structural modifications.^{5–7} For instance, the propargyl functional groups at the 3rd and 4th positions on CXL035 (**2**) and CXL055 (**3**) are critical—replacing either propargyl functional group with an ethyl or a propyl functional group would result in 5–20 fold loss of cytotoxicity. The 3', 4' and 5' positions of the aryl group at the 6th position of the chromene can only accommodate small functional groups, such as methoxy or methylamino groups. Nevertheless, the most potent lead compound (**3**) only showed high nanomolar potency. Further optimization therefore is needed to improve the potency and/or drug properties, which will facilitate future translational development.

This study reported our extended SAR studies at the 4th and 6th position on the 4*H*-chromene based CXL candidates with respect to the cellular cytotoxicity and selectivity towards a multi-drug resistant cancer cell line—HL60/MX2. The results identified additional structural determinants to guide future optimization.

2. Results and discussion

2.1. Rational design of candidates at the 4th position

Our early CXL candidates typically contain two ester functional groups at the 3rd and 4th positions of the chromene system. While different alkoxy functional groups at these positions have showed sharp SARs with respect to cytotoxicity,^{6,7} such ester functional groups may be metabolically labile that would hinder future translational development. In this study, we therefore designed analogs **4–9** with the ester functional group at the 4th position replaced by a ketone moiety or an alcohol functional group, whose cytotoxicity can be directly compared to **1** and **2**, respectively (Fig. 2).

Compounds **4**, **6**, and **8** were synthesized as shown in Scheme 1. The key intermediate chalcone, **11**, was easily accessible via a base-catalyzed aldol condensation of **10** with the corresponding acetophenone in about 70% yield. **11** upon treatment with ethyl cyanoacetate and the preformed sodium alkoxide furnished the desired target compounds **4** or **6** in 50% yield. The alcohol (**8**) was obtained via sodium borohydride reduction of ketone **6** in 90% yield. Compounds **5**, **7**, and **9** were synthesized following similar procedures.

2.2. Rational design of conformation-constrained analogs **12** and **13** at the 6th position

Our earlier lead candidates also all have an aromatic substitution at the 6th position of the 4*H*-chromene, which could adopt different conformations. We hypothesized that some of the conformations may account for most of the cytotoxicity while other conformations may compromise such an activity. We therefore designed analogs **12** and **13** that would force the conformations at the two extreme cases (Fig. 3). Their bioactivity in comparison to **2** and **3**, respectively, may help define the optimal conformations for cytotoxicity and selectivity.

Synthesis of **12** started from 7-hydroxycoumarin (**14**, Scheme 2), which formed the phenolic ether intermediate **15** upon base deprotonation of **14** followed by nucleophilic substitution of an alkyl bromide. **15** was cyclized to generate **16** as the major product following a reported procedure.¹⁴ Compound **12** was obtained in decent yield from **16** via the standard method.⁷

Compound **13** was synthesized as shown in Scheme 3. With slight modifications of the reported procedure,¹⁵ coumarin **18** was obtained. Activation of phenol followed by Suzuki coupling with protected 3-aminoboronic acid furnished **20** in good yield. Upon Boc deprotection, final compound **13** was obtained by following the standard method.⁷

2.3. Cytotoxicity comparison in HL60 and HL60/MX2 cancer cells

HL60 and HL60/MX2 have been well-characterized before that HL60/MX2 reveals multi-drug resistance phenotype and it overexpresses SERCA2 and SERCA3.^{5,11} Our previous data also showed that CXL compounds typically showed preferential cytotoxicity towards HL60/MX2 despite its multi-drug resistance nature towards standard therapies in comparison to the parental HL60 cancer cells. The cytotoxicity and selectivity of the newly synthesized candidates were evaluated in these two cell lines following our established methods.⁵

As shown in Table 1, replacing the ester moiety at the 4th position in **1** and **2** with either an aryl (**4** and **6**) or an alkyl (**5** and **7**) ketone moiety resulted in significant loss of cytotoxicity in both parental and multi-drug resistant leukemia cells, suggesting that the ester functional group at this position may be critical for drug-target interactions. It is also possible that the free acid of the previous CXL compounds might be the active form and the esters serve as prodrugs so that analogs **4–7** are less potent. Reducing the ketone moiety to alcohol (analog **8** and **9**) resulted in further loss of potency in both parental and drug resistant leukemia cell lines. It is possible that increase in flexibility (alcohol vs ketone) at this position is not optimal, which would be consistent with the results of our previous studies.⁶ We have observed that the increase in flexibility at the 3rd and 4th positions typically leads to reduced potency; the propargyl ester compounds were significantly more potent than the propenyl

esters and the propyl esters were the least active. These modifications, however, had a minimal effect on the selectivity between HL60 and HL60/MX2 cells that these new analogs all showed moderate preferential cytotoxicity towards HL60/MX2 cells.

When the two conformation-constrained candidates were evaluated (Table 2), we were surprised by the fact that both analogs were significantly less cytotoxic in comparison to their respective controls. These results suggest that none of the two extreme conformations are the key cytotoxic conformer and that flexibility between the two aromatic rings may be necessary for the compound to effectively interact with its cellular target(s). On the other hand, these two conformational constraints introduced exactly opposite effects on the selectivity of these compounds towards drug resistant HL60/MX2 cells. **13**, which has a methyl substitution at the 5th and 7th positions of the 4*H*-chromene that prohibits the two aryl rings in the same planar, showed no preferential cytotoxicity towards HL60/MX2 at all. Indeed HL60/MX2 showed moderate resistance to **13** in comparison to the parental HL60 cells. **12**, on the other hand, although less cytotoxic towards both HL60 and HL60/MX2 cells in comparison to **2**, showed the highest selectivity towards HL60/MX2. These data suggest that the conformation with both aryl rings in the same planar would improve the selectivity towards the multi-drug resistant HL60/MX2.

3. Conclusions

From these studies, it is clear that an ester functional group at the 4th position and conformational flexibility at the 6th position of the 4*H*-chromene are critical for its cytotoxicity. The conformation at the 6th position also dictates the compound's selective cytotoxicity towards drug resistant cancer cells. These new analogs, although not as cytotoxic as our earlier lead candidates, provide further evidence that 4*H*-chromene is a promising core structure for cancer therapy development towards drug resistant cancer and furnish additional information for future optimization. The conformation-restricted candidates, **12** and **13**, may also serve as useful chemical probes to characterize the molecular basis responsible for the preferential cytotoxicity of **12** towards the multi-drug resistant HL60/MX2 cancer cells.

4. Materials and methods

4.1. Chemistry

All commercial reagents and anhydrous 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 (¹H and ¹³C) spectra were recorded and calibrated using an internal reference. ESI mode mass spectra were recorded on a Bruker BiotofII mass spectrometer. All compounds synthesized are racemic mixtures and are more than 95% pure, as per HPLC analysis.

4.1.1. Synthesis of (*E*)-3-(4-hydroxy-3',5'-dimethoxy-[1,1'-biphenyl]-3-yl)-1-phenylprop-2-en-1-one (11)—To a stirred solution of compound **10** (3.95 mmol) in ethanol (15 mL) was added acetophenone or ketone (3.95 mmol) followed by KOH (3.95 mmol) at room temperature. The reaction mixture was stirred at room temperature for 12 h. After completion of reaction, the solvent was removed under reduced pressure. Followed by the addition of dil HCl (5 mL) at 0 °C, the product was extracted using EtOAc (3 × 15 mL). The combined organic layer was dried over anhydrous Na₂SO₄ with the solvent removed under vacuo to afford the crude chalcone **11**, which was purified by column chromatography on silica gel using EtOAc in Hexanes (50–60%) as the eluent to give pure compound **11** (0.97 g, 70%) as a light yellow solid. TLC (EtOAc/Hexane = 1:1) *R_f* = 0.35, ¹H NMR (400 MHz, CDCl₃): δ 8.24 (1H, d, *J* = 15.5 Hz), 8.08 (2H, d, *J* = 6.5 Hz), 7.80–7.77 (2H, m), 7.61 (2H, d, *J* = 6.5 Hz), 7.52–7.50 (3H, m), 7.02 (1H, br s), 6.72 (2H, s), 6.48 (1H, s), 3.88 (6H, s); ¹³C NMR (100 MHz, CDCl₃): δ 192.0, 161.1, 160.9, 155.6, 142.5, 140.9, 138.2, 134.0, 132.8, 132.6, 130.6, 128.7, 128.6, 127.9, 122.9, 122.3, 117.0, 105.2, 98.8, 55.4, 54.5, 29.7, 27.8; ESI-MS (positive): *m/z* 361.1 (M+H)⁺.

4.1.2. Ethyl 2-amino-6-(3,5-dimethoxyphenyl)-4-(2-oxo-2-phenylethyl)-4*H*-chromene-3-carboxylate (4)—Yield: 46% as a light yellow solid, TLC (EtOAc/Hexane = 1:3) *R_f* = 0.3, ¹H NMR (400 MHz, CDCl₃): δ 7.91 (2H, d, *J* = 8.0 Hz), 7.49–7.53 (1H, m), 7.35–7.44 (4H, m), 7.0 (1H, d, *J* = 8.4 Hz), 6.58 (2H, d, *J* = 2.0 Hz), 6.43 (1H, d, *J* = 2.4 Hz), 4.60 (1H, dd, *J* = 7.6, 4.0 Hz), 4.10–4.21 (2H, m), 3.84 (6H, br s), 3.32 (1H, dd, *J* = 15.6, 4.4 Hz), 3.21 (1H, dd, *J* = 15.6, 8.4 Hz) 1.25 (3H, t, *J* = 2.4 Hz); ¹³C NMR (100 MHz, CDCl₃): δ 198.6, 169.0, 161.5, 160.9, 149.5, 142.5, 137.4, 137.2, 132.9, 128.6, 128.5, 128.2, 127.4, 126.38, 126.34, 116.0, 105.2, 105.1, 99.3, 77.47, 60.2, 59.56, 55.39, 48.5, 45.1, 31.3, 21.5, 14.5; ESI-MS *m/z*: (Calcd for C₂₈H₂₈NO₆: 474.1917); Found: 474.1906 (M+H)⁺.

4.1.3. Ethyl 2-amino-6-(3,5-dimethoxyphenyl)-4-(2-oxopentyl)-4*H*-chromene-3-carboxylate (5)—Yield: 45% as a light yellow solid, TLC (EtOAc/Hexane = 1:3) *R_f* = 0.39, ¹H NMR (400 MHz, CDCl₃): δ 7.52 (1H, br s), 7.42 (1H, d, *J* = 8.4 Hz), 7.01 (2H, d, *J* = 6.0 Hz), 6.65 (1H, br s), 6.51 (1H, br s), 6.29 (2H, br s), 4.21–4.23 (1H, m), 4.10–4.13 (2H, m), 3.81 (6H, s), 2.67–2.69 (2H, m), 2.22–2.26 (2H, m), 1.52–1.56 (2H, m), 1.21–1.20. (3H, m), 0.81 (3H, t, *J* = 3.6 Hz); ¹³C NMR (100 MHz, CDCl₃): δ 209.4, 169.0, 161.0, 156.4, 142.5, 137.4, 127.3, 126.6, 126.2, 116.0, 107.2, 105.2, 99.2, 96.4, 78.5, 59.5, 55.4, 52.0, 45.9, 45.7, 30.1, 29.6, 17.0, 14.5, 13.6; ESI-MS *m/z*: (Calcd for C₂₅H₃₀NO₆: 440.2073); Found: 440.2070 (M+H)⁺.

4.1.4. Prop-2-yn-1-yl 2-amino-6-bromo-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4*H*-chromene-3-carboxylate (6)—Yield: 46% as a light yellow solid, 84% purity based on NMR integration, TLC (EtOAc/Hexane = 1:3) *R_f* = 0.33, ¹H NMR (400 MHz, CDCl₃): δ 7.93 (1H, d, *J* = 8.4 Hz), 7.77 (1H, d, *J* = 7.2 Hz), 7.43–7.33 (4H, m), 7.12 (1H, d, *J* = 8.4 Hz), 7.00 (1H, d, *J* = 8.4 Hz), 6.58 (2H, d, *J* = 2.0 Hz), 6.43–6.42 (1H, m), 4.74 (2H, dd, *J* = 6.0, 2.4 Hz), 4.57 (1H, dd, *J* = 7.6, 3.6 Hz), 3.82 (6H, br s), 3.37 (1H, dd, *J* = 15.2, 4.4 Hz), 3.26 (1H, dd, *J* = 15.2, 8.4 Hz), 2.44 (1H, t, *J* = 2.4 Hz); ¹³C NMR (100 MHz, CDCl₃): δ 161.0, 149.2, 142.4, 137.1, 132.9, 128.8, 128.5, 128.3, 128.0, 127.4, 126.5, 125.9, 116.1, 105.3, 105.1, 99.3, 87.5, 76.6, 74.3, 66.8, 55.5, 55.4, 54.6, 51.1, 46.0,

48.2, 31.2, 29.7, 25.8; ESI-MS m/z : (Calcd for $C_{29}H_{26}NO_6$: 484.1760); Found: 484.1754 ($M+H$)⁺.

4.1.5. Prop-2-yn-1-yl 2-amino-6-(3,5-dimethoxyphenyl)-4-(2-oxopentyl)-4H-chromene-3-carboxylate (7)—Yield: 44%, TLC (EtOAc/Hexane = 1:3) R_f = 0.36, 1H NMR (400 MHz, $CDCl_3$): δ 7.45 (1H, br s), 7.37 (1H, dd, J = 8.8, 2.2 Hz), 7.01 (1H, d, J = 8.4 Hz), 6.65 (2H, d, J = 8.4 Hz), 6.44 (2H, d, J = 1.6 Hz), 4.79 (2H, dq, J = 15.6, 2.4 Hz), 4.38–4.41 (1H, m), 4.10–4.13 (2H, m), 3.86 (6H, s), 2.70–2.81 (2H, m), 2.44 (1H, t, J = 2.0 Hz), 2.18–2.30 (2H, m), 1.49 (2H, q, J = 6.8 Hz), 0.81 (3H, t, J = 1.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 209.4, 167.9, 162.3, 161.0, 149.2, 142.4, 137.6, 127.3, 126.4, 126.3, 116.0, 105.2, 99.2, 78.7, 76.6, 76.5, 74.2, 74.1, 55.4, 51.8, 51.1, 51.0, 45.6, 30.0, 17.0, 13.6; ESI-MS m/z : (Calcd for $C_{26}H_{28}NO_6$: 450.1917); Found: 450.1910 ($M+H$)⁺.

4.1.6. Prop-2-yn-1-yl 2-amino-6-(3,5-dimethoxyphenyl)-4-(2-hydroxy-2-phenylethyl)-4H-chromene-3-carboxylate (8)—To a stirred solution of compound **6** (0.02 mmol) in methanol (5 mL) was added $NaBH_4$ (0.04 mmol). The reaction mixture was stirred at room temperature under inert atmosphere for 30 min. After the completion of reaction, solvent was removed under reduced pressure. Followed by the addition of dil HCl (5 mL) at 0 °C, the product was extracted using EtOAc (2 × 5 mL). The combined organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated under vacuo to afford the crude alcohol and was purified by column chromatography on silica gel using EtOAc in Hexanes (50–60%) as the eluent to give the pure alcohol **8** as a light yellow oil. Yield: 90%, TLC (EtOAc/Hexane = 1:1) R_f = 0.36, obtained as a 1:1 mixture of two sets of diastereomers based on 1H NMR. 1H NMR (400 MHz, $CDCl_3$): δ 7.44–7.34 (11H, m), 7.04 (1H, d, J = 8.0 Hz), 6.62–6.60 (4H, m), 6.43 (1H, s), 5.44 (1H, d, J = 12 Hz), 4.11–4.07 (1H, m), 3.81 (6H, s), 2.76 (1H, d, J = 12 Hz), 2.03 (1H, m); ESI-MS m/z : (Calcd for $C_{29}H_{27}NO_6$: 486.1917); Found: 486.1906 ($M+H$)⁺.

4.1.7. Prop-2-yn-1-yl 2-amino-6-(3,5-dimethoxyphenyl)-4-(2-hydroxypentyl)-4H-chromene-3-carboxylate (9)—Yield: 65% as a light yellow oil, 85% purity based on HPLC integration, TLC (EtOAc/Hexane = 1:1) R_f = 0.36, obtained as a 1:1 mixture of two sets of diastereomers based on 1H NMR. 1H NMR (400 MHz, $CDCl_3$): δ 7.41–7.32 (2H, m), 7.02–6.93 (2H, m), 6.64–6.63 (3H, m), 6.45–6.40 (2H, m), 4.57–4.54 (1H, d, J = 15 Hz), 4.13–4.10 (1H, m), 3.92–3.90 (1H, m), 3.84 (6H, s), 2.25–2.17 (2H, m), 1.81–1.73 (2H, m), 1.43–1.41 (2H, m), 0.98–0.94 (3H, m); ESI-MS m/z : (Calcd for $C_{26}H_{30}NO_6$: 452.2073); Found: 452.2059 ($M+H$)⁺.

4.1.8. 7-((2-Bromo-4,6-dimethoxybenzyl)oxy)-2H-chromen-2-one (15)—Yield: 94%, TLC (EtOAc/Hexane = 1:3) R_f = 0.34, 1H NMR (500 MHz, $CDCl_3$): δ 7.56 (1H, d, J = 9.5 Hz), 7.30–7.18 (1H, m), 6.90 (1H, d, J = 2.0 Hz), 6.85 (1H, d, J = 2.0 Hz), 6.84 (1H, d, J = 2.0 Hz), 6.70 (1H, d, J = 2.5 Hz), 6.37 (1H, d, J = 2.0 Hz), 6.17 (1H, d, J = 9.5 Hz), 5.11 (2H, br s), 3.75 (3H, s), 3.73 (3H, s); ^{13}C NMR (125 MHz, $CDCl_3$): δ 162.4, 161.5, 161.3, 159.9, 155.8, 143.5, 128.6, 127.4, 116.2, 113.4, 112.9, 112.5, 109.4, 101.8, 98.2, 64.6, 56.0, 55.6; ESI-MS (positive): m/z 391.1 ($M+H$)⁺.

4.1.9. 9,11-Dimethoxy-2*H*,8*H*-benzo[*c*]pyrano[2,3-*f*]chromen-2-one (16a)—

Yield: 16%, TLC (EtOAc/Hexane = 1:3) R_f = 0.24, ^1H NMR (500 MHz, CDCl_3): δ 7.82 (1H, s), 7.69 (1H, d, J = 9.5 Hz), 7.33 (1H, d, J = 8.5 Hz), 6.96 (1H, d, J = 8.5 Hz), 6.53 (1H, s), 6.33 (1H, d, J = 9.5 Hz), 5.19 (2H, s), 3.95 (3H, s), 3.87 (3H, s); ^{13}C NMR (125 MHz, CDCl_3): δ 160.4, 159.3, 155.4, 152.3, 144.4, 138.5, 128.4, 115.0, 114.5, 113.9, 112.9, 111.8, 109.7, 103.0, 99.0, 97.8, 63.4, 55.6; ESI-MS (positive): m/z 311.1 ($\text{M}+\text{H}$) $^+$.

4.1.10. 2,4-Dimethoxy-5*H*,9*H*-benzo[*c*]pyrano[3,2-*g*]chromen-9-one (16)—

Yield: 48%, TLC (EtOAc/Hexane = 1:3) R_f = 0.25, ^1H NMR (500 MHz, CDCl_3): δ 7.69 (1H, d, J = 10 Hz), 7.67 (1H, s), 6.88 (1H, s), 6.77 (1H, s), 6.44 (1H, s), 6.27 (1H, d, J = 9.5 Hz), 5.21 (2H, s), 3.89 (3H, s), 3.84 (3H, s); ^{13}C NMR (125 MHz, CDCl_3): δ 161.3, 160.8, 158.3, 155.8, 143.4, 129.8, 127.5, 122.5, 119.9, 116.2, 113.8, 112.5, 109.5, 105.2, 101.8, 98.1, 63.7, 55.6; ESI-MS (positive): m/z 311.1 ($\text{M}+\text{H}$) $^+$.

4.1.11. Prop-2-yn-1-yl 9-amino-2,4-dimethoxy-11-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-5*H*,11*H*-benzo[*c*]pyrano[3,2-*g*] chromene-10-carboxylate (12)—

Yield: 44% as a yellow solid, TLC (EtOAc/Hexane = 1:3) R_f = 0.14, ^1H NMR (500 MHz, CDCl_3): δ 7.53 (1H, s), 6.74 (1H, d, J = 2 Hz), 6.58 (1H, s), 6.39 (1H, d, J = 2 Hz), 5.18–5.06 (2H, m), 4.78 (2H, s), 4.60 (2H, s), 4.34–4.36 (1H, m), 3.89 (3H, s), 3.82 (3H, s), 2.76–2.72 (1H, m), 2.68–2.64 (1H, m), 2.47 (1H, s), 2.37 (1H, s); ^{13}C NMR (125 MHz, CDCl_3): δ 170.9, 167.9, 162.1, 160.6, 156.0, 154.6, 150.3, 131.0, 123.2, 119.7, 118.6, 111.9, 104.7, 98.2, 97.6, 78.7, 77.7, 76.1, 74.9, 74.3, 63.3, 55.5, 51.8, 51.1, 43.5, 30.6, 29.7; ESI-MS m/z : (Calcd for $\text{C}_{27}\text{H}_{24}\text{NO}_8$: 490.1502); Found: 490.1492 ($\text{M}+\text{H}$) $^+$.

4.1.12. 6-Hydroxy-5,7-dimethyl-2*H*-Chromen-2-one (18)—Yield: 94%, TLC (EtOAc/Hexane = 2:3) R_f = 0.29, ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 8.51 (1H, s), 8.12 (1H, d, J = 9.6 Hz), 7.01 (1H, s), 6.33 (1H, d, J = 9.6 Hz), 2.32 (3H, s), 2.24 (3H, s); ^{13}C NMR (100 MHz, CDCl_3): δ 160.1, 149.4, 147.7, 141.8, 131.2, 121.3, 115.9, 114.9, 114.2, 17.3, 11.3. ESI-MS (positive): m/z 191.1 ($\text{M}+\text{H}$) $^+$

4.1.13. 5,7-Dimethyl-2-oxo-2*H*-Chromen-6-yl trifluoromethanesulfonate (19)—

Yield: 91%, TLC (EtOAc/Hexane = 2:3) R_f = 0.89, ^1H NMR (400 MHz, CDCl_3): δ 7.89 (1H, d, J = 10 Hz), 7.11 (1H, s), 6.47 (1H, d, J = 12 Hz), 2.53 (3H, s), 2.48 (3H, s). ^{19}F NMR (376 MHz, CDCl_3): δ -73.19 (s, CF_3). ESI-MS (positive): m/z 323.0 ($\text{M}+\text{H}$) $^+$.

4.1.14. *t*-Butyl(3-(5,7-dimethyl-2-oxo-2*H*-chromen-6-yl) phenyl)carbamate (20)

—Yield: 78%, TLC (EtOAc/Hexane = 1.5:3.5) R_f = 0.72, ^1H NMR (400 MHz, CDCl_3): δ 7.94 (1H, d, J = 8.8 Hz), 7.41–7.37 (2H, m), 7.2 (1H, s), 7.07 (1H, s), 6.67 (1H, d, J = 6.8 Hz), 6.60 (1H, s), 6.39 (1H, d, J = 9.6 Hz), 2.19 (3H, s), 2.10 (3H, s), 1.51 (9H, s). ^{13}C NMR (100 MHz, CDCl_3): δ 161.2, 153.8, 152.8, 146.7, 141.8, 141.2, 140.8, 139.0, 138.6, 133.9, 129.5, 124.0, 119.1, 117.6, 115.7, 115.5, 115.1, 115.0, 80.8, 28.5, 21.9, 16.3.

4.1.15. 6-(3-Aminophenyl)-5,7-dimethyl-2*H*-chromen-2-one (21)—

Yield: 96%, TLC (EtOAc/Hexane = 3:2) R_f = 0.11, ^1H NMR (400 MHz, CDCl_3): δ 7.95 (1H, d, J = 10 Hz), 7.23 (1H, t, J = 8 Hz), 7.07 (1H, s), 6.69 (1H, m), 6.48 (1H, d, J = 7.6 Hz), 6.43 (1H, m), 6.38 (1H, d, J = 1 Hz), 3.74 (2H, br s), 2.21 (3H, s), 2.12 (3H, s). ^{13}C NMR (100 MHz,

CDCl₃): δ 161.3, 153.7, 146.8, 141.8, 141.2, 141.0, 139.1, 133.8, 129.8, 119.6, 115.9, 115.7, 115.4, 115.0, 114.1, 21.8, 16.2. ESI-MS (positive): m/z 266.1 (M+H)⁺.

4.1.16. Prop-2-yn-1-yl 2-amino-6-(3-aminophenyl)-5,7-dimethyl-4-(2-oxo-2-(prop-2-yn-1-yloxy)ethyl)-4H-chromene-3-carboxylate (13)—Yield: 39% as a light yellow solid, TLC (EtOAc/Hexane = 1:3) R_f = 0.13, ¹H NMR (400 MHz, CDCl₃): δ 7.22–7.17 (1H, m), 6.75 (1H, s), 6.68–6.66 (1H, m), 6.52–6.38 (4H, m), 4.75 (2H, d, J = 2.4 Hz), 4.59 (2H, t, not resolved well), 4.37–4.35 (1H, m), 3.72 (2H, br s), 2.71–2.66 (1H, m), 2.58–2.52 (1H, m), 2.5–2.41 (2H, m), 2.07 (3H, s), 1.99 (3H, s). ¹³C NMR (100 MHz, CDCl₃): δ 170.8, 168.0, 162.8, 149.3, 146.7, 142.1, 139.4, 136.0, 133.9, 129.8, 121.1, 119.6, 115.9, 114.5, 113.7, 78.9, 78.0, 76.4, 75.1, 74.6, 52.0, 51.1, 41.4, 29.7, 20.9, 16.6. ESI-MS m/z : (Calcd for C₂₆H₂₅N₂O₅: 445.1763); Found: 445.1630 (M+H)⁺.

4.2. In vitro cytotoxicity

HL60 and HL60/MX2 were purchased from ATCC. Both cell lines were maintained in RPMI 1640 (Invitrogen) medium supplemented with 10% FBS and 1% penicillin–streptomycin (antibiotic) at 37 °C with 5% CO₂ in air atmosphere. The in vitro cytotoxicity of the compounds was determined based on their ability to inhibit the growth of HL60 and HL60/MX2 cells. In brief, HL60 or HL60/MX2 cells were plated in a 96-well plate (at a density of 1×10^4 cells/well) and were treated with the test compounds in a series of dilutions with 1% DMSO in the final cell medium in triplicate (cells treated with medium containing 1% DMSO served as controls). After a 48-h treatment, the relative cell viability in each well was determined by using Cell Titer-Blue Cell Viability Assay kit according to manufacturer's protocol (Promega). The IC₅₀ of each candidate was determined by fitting the relative viability of the cells to the drug concentration by using a dose–response model in Prism program from GraphPad Software, Inc. (San Diego, CA). Each compound was evaluated with at least three biological repeats on different days. Data were reported as mean IC₅₀ $\pm$ SD. Statistical significance was determined by using the two-tailed Student *t*-tests.

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Figure 1.

Recently published CXL lead compounds—CXL017 (1), CXL037 (2) and CXL055 (3).^{5–7}

Figure 2.
Rational design of analogs 4–9 to determine the importance of the ester functional group at the 4th position.

Figure 3. Rational design of **12** and **13** to determine the importance of the conformational flexibility at the 6th position.

Scheme 1.
Synthesis of 4-keto or hydroxyl analogs—4, 6 and 8.

Scheme 2.
Synthesis of conformation-constrained analog 12.

Scheme 3.
Synthesis of conformation-constrained analog **13**.

Table 1

Cytotoxicity against HL60 and HL60/MX2 cells

	IC ₅₀ (Mean ± SD in µM)		Ratio ^a	<i>p</i> ^b
	HL60	HL60/MX2		
1	5.4 ± 1.7	1.7 ± 0.6	0.32	<0.0001
4	109 ± 10	41 ± 4	0.38	0.01
5	27.3 ± 5.0	11.7 ± 1.3	0.43	0.002
2	1.16 ± 0.21	0.86 ± 0.10	0.74	>0.05
6	7.3 ± 1.5	5.9 ± 0.7	0.81	>0.05
7	6.9 ± 0.5	5.4 ± 0.6	0.78	>0.05
8	100±5	54±13	0.54	0.011
9	46.6 ± 2.5	35.8 ± 2.4	0.77	0.004

^a The ratio of IC₅₀s between HL60/MX2 and HL60.^b Statistical analysis was performed with two-tailed Student t test between HL60 and HL60/MX2.

Table 2

Cytotoxicity against HL60 and HL60/MX2 cells

	IC ₅₀ (Mean ± SD in µM)		Ratio ^a	<i>p</i> ^b
	HL-60	HL-60/MX2		
2	1.16 ± 0.21	0.86 ± 0.10	0.74	>0.05
12	41.9 ± 10.7	7.4 ± 1.1	0.18	0.0001
3	1.73 ± 0.59	0.54 ± 0.06	0.31	0.009
13	50.5 ± 6.3	82.2 ± 1.0	1.63	0.0013

^a The ratio of IC₅₀s between HL60/MX2 and HL60.^b Statistical analysis was performed with two-tailed Student *t* test between HL60 and HL60/MX2.