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Discovery of 4-aryl-4*H*-chromenes as a new series of apoptosis inducers using a cell- and caspase-based HTS assay. Part 5: Modifications of the 2- and 3-positions

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Abstract—As a continuation of our efforts to discover and develop apoptosis inducing 4-aryl-4*H*-chromenes as novel anticancer agents, we explored modifications at the 2- and 3-positions. It was found that replacement of the 3-cyano group by an ester, including methyl and ethyl ester, resulted in >200-fold reduction of activity. Conversion of the 2-amino group into an amide or urea resulted in 4- to 10-fold drop of activity. Similarly, converting the 2-amino group into a hydrogen resulted in 4- to 10-fold reduction of activity. Compound **3d** was highly active with an EC_{50} value of 29 nM and a GI_{50} value of 6 nM in T47D cells. Importantly, the 2-H analog **3d** was found to be much more stable under acidic conditions compared to the 2-NH_2 analog **3b**, suggesting that 2-H analogs might have better bioavailability than the 2-NH_2 analogs.

Apoptosis is known to play a vital role in the elimination of damaged or excess cells. Defects in the apoptosis pathways are one of the hallmarks of cancers that lead to uncontrollable tumor cell growth, as well as tumor resistance to chemotherapeutic treatment.[1] The pathways of apoptosis involve a cascade of initiator and effector caspases. Caspase-3 is known to be the main executioner of apoptosis through cleavage of protein substrates that leads to irreversible cell death.[2] Since many of the clinically useful cytotoxic agents are known to induce apoptosis in cancer cells,[3] we have been interested in the discovery and development of apoptosis inducers as potential anticancer agents,[4] and have therefore developed a cell-based high throughput-screening assay for apoptosis inducers, using our proprietary fluorescent caspase-3 substrate.[5]

We have recently reported the discovery and structure–activity relationship (SAR) of 4-aryl-4*H*-chromenes as a new series of potent apoptosis inducers using our cell- and caspase-based high-throughput screening (HTS) assays. These compounds were found to be tubulin inhibitors, binding at or close to the binding site of colchicine, possessing vascular targeting activity and showing high activity in several anticancer animal models.[6,7] From our screening hit 2-amino-3-cyano-7-dimethylamino-4-(3-methoxy-4,5-methylenedioxyphenyl)-4*H*-chromene (**1a**), we have identified 2-amino-4-(3-bromo-4,5-dimethoxyphenyl)-3-cyano-7-dimethylamino-4*H*-chromene (**2a**) as a lead compound.[8] Additional SAR studies showed that cyclization of the 7,8-positions[9] into a ring structure led to potent compounds, such as 2-amino-4-(3-bromo-4,5-dimethoxyphenyl)-3-cyano-4,7-dihydropyrrolo[2,3-*h*]chromene (**3a**).[10] Introduction of a methyl group at the 7-position of the pyrrolo ring led to highly potent compounds such as 2-amino-4-(3-bromo-4,5-dimethoxyphenyl)-3-cyano-4,7-dihydro-7-methylpyrrolo[2,3-*h*]chromene (**3b**)[11] with EC_{50} values in the 2–3 nM range (Chart 1).

It has been reported that a series of structurally related substituted 2-amino-3-cyano-4-phenyl-4*H*-naphtho[1,2-

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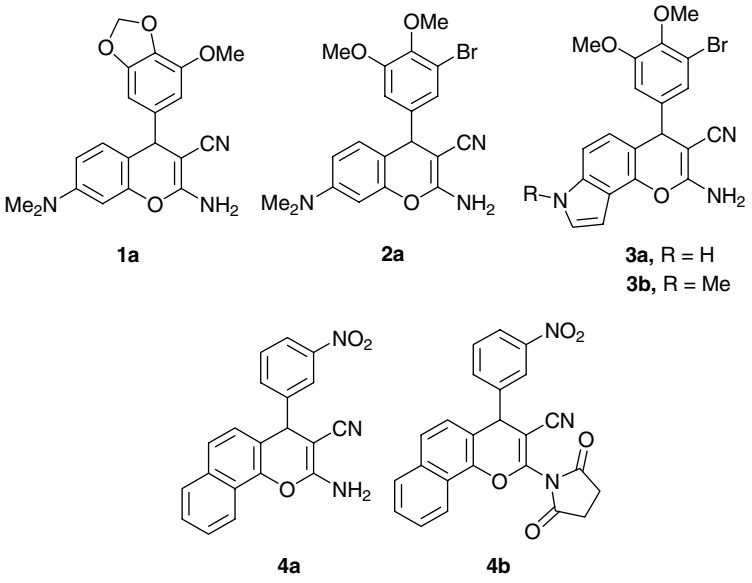
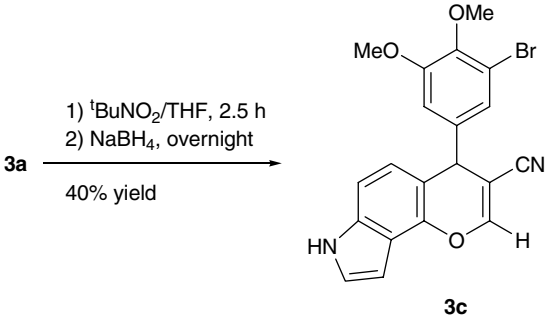


Chart 1.

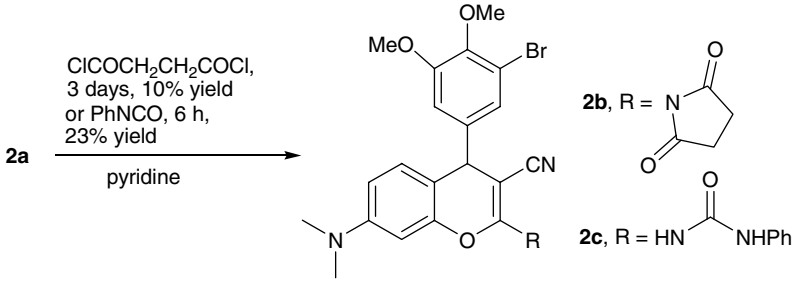
b]pyrans such as compound **4a** blocked the synthesis and not the activity of matrix-degrading metalloproteases, and thus might be useful for the prevention of cartilage destruction associated with several degenerative diseases of the articular joint such as rheumatoid arthritis.[12] Compound **4a** was found to be unstable in an acidic solution (50/50/0.1, v/v/v acetonitrile/water/TFA), thus limiting its oral bioavailability. The 2-amino group was found to be the cause of the instability of **4a** and conversion of the amino group into a succinimido group (**4b**) rendered the compound stable under acidic conditions.[13] Although most of the clinically used tubulin interacting chemotherapeutics including paclitaxel and doxetaxel are administered by iv injection, it is desirable to have orally active antitubulin drugs. Currently there are several orally active tubulin interacting agents in clinical development, including ABT-751[14] and indibulin,[15] which are in phase II and phase I clinical trials, respectively. We therefore explored the modification of the 2-amino group and the 3-cyano group, both as a continuation of our SAR study as well as to improve stability of 4-aryl-4*H*-chromenes under acidic conditions. We now wish to report the SAR at the 2 and 3-positions of 4-aryl-4*H*-chromenes.

The 2-succinimido 4*H*-chromene **2b** was prepared via reaction of amine **2a** with succinyl chloride in pyridine. Similarly, 2-ureido **2c** was prepared via reaction of **2a** with phenyl isocyanate in pyridine (Scheme 1).

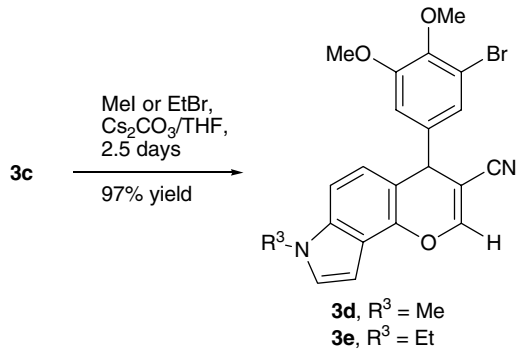
2-H analogs were prepared in a one-pot reaction via diazotization of the amino group using *t*-butylnitrite (*t*-$BuNO_2$)[16] in THF, followed by reduction of the diazonium group using $NaBH_4$, as shown for the preparation of **3c** (Scheme 2).[17] 2-H analog **2d** was prepared



Scheme 2.



Scheme 1.



Scheme 3.

from the corresponding amino compound **2a** using a similar procedure. 2-H analogs **3d–3e** were prepared, respectively, from reaction of **3c** with iodomethane and bromoethane in THF in the presence of Cs_2CO_3 (Scheme 3).[18]

The 3-ester **3f** was prepared as shown in Scheme 4 by a one-pot reaction of 3-bromo-4,5-dimethoxybenzaldehyde, 1*H*-indol-4-ol, and methyl 2-cyanoacetate in ethanol in the presence of piperidine, using a procedure similar to the preparation of 3-cyano analogs such as **3a**.[8] Ester **3g** was prepared similarly using ethyl 2-cyanoacetate instead of methyl 2-cyanoacetate (Scheme 4).

The apoptosis inducing activity of these 4-aryl-4*H*-chromenes was measured by our cell- and caspase-based HTS assay[19] in human breast cancer cells T47D, human colon cancer cells HCT116, and hepatocellular carcinoma cancer cells SNU398. The results are summarized in Table 1. Starting from our lead compound **2a**, we first explored the effects of converting the 2-amino group into an imide and urea group (Table 1). Succinimide **2b** and urea **2c** were 13- and 4-fold less active than **2a**, suggesting that the amino group might be important for activity or the 2-position is size limited.

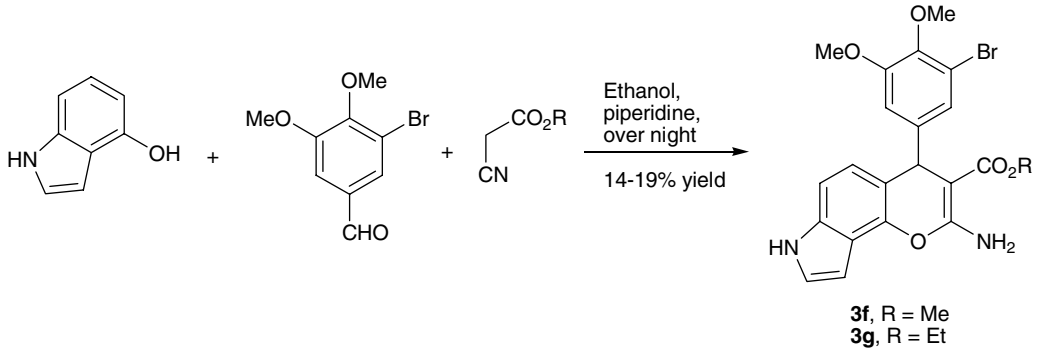
We then explored the conversion of the 2-amino group into a 2-H group. Compound **2d** was about 4-fold less active than **2a**, suggesting that the 2-amino group is important but not essential for the apoptosis inducing activity of these compounds. Similarly, 2-H analogs **3c–3e** were found to have good activity. Compound **3d**, with an EC_{50} of 29 nM, is still highly active but about 10-fold less active than the corresponding 2-NH_2 analog **3b**.

We also explored the replacement of the 3-CN group by an ester group. The 3-methyl ester **3f** and 3-ethyl ester **3g** were found to have low activity with EC_{50} values of 1–3 μM, which is >200-fold less active than the corresponding 3-CN analog **3a**. These data suggest that the 3-CN group is important for the activity and that there might be a size limited pocket around the 3-position.

The activities of these compounds toward the colon cancer cells HCT116 and the hepatocellular carcinoma cancer cells SNU398 were roughly parallel to their activity toward T47D cells. In general, both were slightly less sensitive to these compounds than T47D cells in this assay as indicated by the slightly higher EC_{50} value.

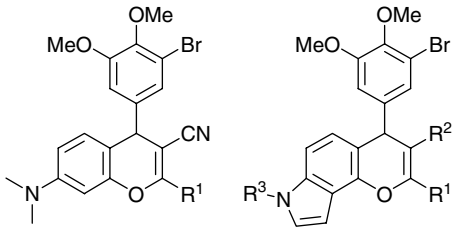
Selected compounds were also tested by the traditional inhibition of cell growth (GI_{50}) assay to confirm that the active compounds can inhibit tumor cell growth. The growth inhibition assays in T47D, HCT116, and SNU398 cells were run in a 96-well microtiter plate as described previously.[19] The GI_{50} are summarized in Table 2. Compound **3d** was found to be highly active with GI_{50} values of 4–6 nM in the three cell lines tested. Similar to what is observed in the caspase activation assay, compound **3g** was found to have low activity in the growth inhibition assay, suggesting a good correlation between these two assays.[17]

Since compound **3d** was found to have good activity in both the caspase activation assay and the growth inhibition assay, and the 2-NH_2 group has been reported to contribute to the instability of these compounds under acidic conditions,[13] we compared the stability of 2-H analog **3d** versus 2-NH_2 analog **3b** in acidic solution. When a solution of the 2-amino compound **3b** in DMSO-d_6 and D_2O (v/v:8/2) with a drop of TFA (pH 4) was monitored by ^1H NMR, it was found that after the solution was stored at room temperature overnight, all the NMR signals became multi-peaks, suggesting that the compound undergoes rapid decomposition under acidic conditions, similar to what had been reported for structurally related compound **4b**.[13] In comparison, a solution of the corresponding 2-H analog **3d** in



Scheme 4.

Table 1. SAR of 2- and 3-positions of 4-aryl-chromenes in the caspase activation assay



Entry	R^1	R^2	R^3	EC_{50}[a] (μM)		
				T47D	HCT116	SNU398
2a	NH_2	NA	NA	0.019 ± 0.004[b]	0.009 ± 0.0004[d]	0.019 ± 0.0007[d]
2b	Succinimido	NA	NA	0.25 ± 0.01	0.42 ± 0.03	0.19 ± 0.03
2c	NHCONHPh	NA	NA	0.068 ± 0.001	0.084 ± 0.008	0.093 ± 0.007
2d	H	NA	NA	0.065 ± 0.002	0.093 ± 0.013	0.077 ± 0.018
3a	NH_2	CN	H	0.005 ± 0.0003[c]	0.014 ± 0.002[d]	0.015 ± 0.001[d]
3b	NH_2	CN	Me	0.002 ± 0.0003[d]	0.003 ± 0.0004[d]	0.002 ± 0.0004[d]
3c	H	CN	H	0.047 ± 0.005	0.066 ± 0.006	0.052 ± 0.003
3d	H	CN	Me	0.029 ± 0.002	0.038 ± 0.003	0.034 ± 0.004
3e	H	CN	Et	0.070 ± 0.014	0.15 ± 0.04	0.087 ± 0.017
3f	NH_2	CO_2Me	H	1.4 ± 0.5	0.66 ± 0.27	1.0 ± 0.4
3g	NH_2	CO_2Et	H	3.2 ± 0.3	3.0 ± 0.2	2.2 ± 0.2

[a] Data are means of three or more experiments and are reported as means ± standard error of the mean (SEM).
[b] Data from Ref. 8.
[c] Data from Ref. 10.
[d] Data from Ref. 11.

Table 2. Inhibition of cell growth of 4-aryl-chromenes

Compound	GI_{50}[a] (μM)		
	T47D	HCT116	SNU398
2d	0.066 ± 0.012	0.051 ± 0.003	0.017 ± 0.001
3c	0.073 ± 0.011	0.053 ± 0.005	0.034 ± 0.014
3d	0.006 ± 0.001	0.006 ± 0.001	0.004 ± 0.001
3g	3.3 ± 0.4	4.3 ± 0.5	1.6 ± 0.1

[a] Data are means of three or more experiments and are reported as means ± standard error of the mean (SEM).

DMSO-d_6/D_2O/TFA under the same conditions was found to be stable after 8 days, indicating that **3d** with a hydrogen at the 2-position was highly stable under acidic conditions. Our results confirmed the early report that the 2-amino group was responsible for the instability of this class of compounds under acidic conditions and suggested that the 2-des-amino analogs offer a straightforward way to address the instability problems, thus improving the bioavailability of this class of potent apoptosis inducers.

We have reported that compound **2a** and related compounds are tubulin inhibitors that bind at or close to the colchicine site of β-tubulin.[6] Compounds **3c** and **3d** were tested in the tubulin polymerization assay. Similar to **2a**, both compounds were found to inhibit tubulin polymerization with IC_{50} values of less than 500 nM. Therefore, converting the 2-amino group into a 2-H group does not change the mechanism of action of these compounds, which is induction of apoptosis through inhibition of tubulin polymerization. In addition, compounds in this series that are less active in the caspase activation assay also were found to be less active in the tubulin polymerization assay, confirming that tubulin inhibition is the main mechanism of action of these substituted chromenes.[20]

In conclusion, we have explored the modification of the 2- and 3-positions of apoptosis inducing 4-aryl-4*H*-chromenes as potential anticancer agents. It was found that replacing the 3-CN group by an ester resulted in a large drop of activity. Modification of the 2-NH_2 group into amide and urea resulted in 4- to 10-fold reduction of activity. Converting the 2-NH_2 group into a 2-H group was found to be relatively well tolerated, with only a 4- to 10-fold reduction of activity. Importantly, the 2-H analog **3d** was found to have good activity with an EC_{50} value of 29 nM and a GI_{50} value of 6 nM in T47D cells. Moreover, **3d** has shown high stability under acidic conditions in comparison to the 2-NH_2 analog **3b**, confirming that the 2-NH_2 group is responsible for the instability of this class of compounds under acidic conditions and suggesting that the 2-H analogs might have improved oral bioavailability.

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17. Experimental procedure for the synthesis of 4-(3-bromo-4,5-dimethoxy-phenyl)-3-cyano-4,7-dihydro-pyrano[2,3-*e*]indole (**3c**). To a solution of 2-amino-4-(3-bromo-4,5-dimethoxy-phenyl)-3-cyano-4,7-dihydro-pyrano[2,3-*e*]indole (0.998 g, 2.34 mmol) in anhydrous THF (40 mL) at −30 °C was added ^{t}butylnitrite (1.00 mL, 7.58 mmol) via a syringe under argon. The light yellow solution was stirred between −20 and −10 °C for 4 h, then warmed up to 0 °C. The reaction mixture was cooled to −10 °C and $NaBH_4$ (0.259 g, 6.84 mmol) was added in one portion. The reaction mixture was warmed up to room temperature slowly and stirred overnight (15 h). The THF solvent was evaporated and the residue was dissolved in EtOAc (100 mL). The mixture was washed with saturated $NaHCO_3$ (15 mL) and brine (15 mL), dried over $MgSO_4$, and evaporated to give a brown residue. It was purified by flash column chromatography (silica gel, EtOAc:hexanes/1:2) to give **3c** as an off-white solid (0.39 g, 40%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.30 (bs, 1H), 7.45 (m, 1H), 7.24 (m, 1H), 7.13 (m, 1H), 6.96 (d, J = 2.1 Hz, 1H), 6.76 (d, J = 1.8 Hz, 1H), 6.70 (d, J = 8.1 Hz, 1H), 6.67 (m, 1H), 4.81 (s, 1H), 3.84 (s, 3H), 3.83 (s, 3H).
18. Experimental procedure for the synthesis of 4-(3-bromo-4,5-dimethoxy-phenyl)-3-cyano-4,7-dihydro-7-methyl-pyrano[2,3-*e*]indole (**3d**). A mixture of 4-(3-bromo-4,5-dimethoxy-phenyl)-3-cyano-4,7-dihydro-pyrano[2,3-*e*]indole (51 mg, 0.12 mmol), iodomethane (23 μL, 0.37 mmol), and cesium carbonate (120 mg, 0.37 mmol) in THF (2 mL) was stirred at room temperature in a sealed tube for 2.5 days. The reaction mixture was diluted with EtOAc (20 mL). The mixture was filtered, and the solid was washed with EtOAc (30 mL). The EtOAc solution was combined, washed with brine (2× 5 mL), dried over $MgSO_4$, and evaporated to give an off-white solid. It was purified by column chromatography (silica gel, EtOAc:hexanes/1:1) to give **3d** as an off-white solid (51 mg, 97%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.43 (m, 1H), 7.07 (d, J = 3.3 Hz, 1H), 7.05 (m, 1H), 6.95 (d, J = 2.1 Hz, 1H), 6.76 (d, J = 2.4 Hz, 1H), 6.72 (d, J = 8.4 Hz, 1H), 6.58 (m, 1H), 4.81 (s, 1H), 3.84 (s, 3H), 3.82 (s, 3H), 3.78 (s, 3H).
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20. For example, compound **3a**, with an EC_{50} value of 5 nM in the caspase activation assay, was found to have an IC_{50} value of 500 nM in the tubulin polymerization assay. In comparison, the 4-phenyl analog of **3a**, 2-amino-4-phenyl-3-cyano-4,7-dihydro-pyrano[2,3-*e*]indole, with an EC_{50} value of 270 nM in the caspase activation assay, was found to have an IC_{50} value of 3000 nM in the tubulin polymerization assay.