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Discovery of 5-(4-hydroxy-6-methyl-2-oxo-2*H*-pyran-3-yl)-7-phenyl-(*E*)-2,3,6,7-tetrahydro-1,4-thiazepines as a new series of apoptosis inducers using a cell- and caspase-based HTS assay

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Abstract—We report the discovery of 5-(4-hydroxy-6-methyl-2-oxo-2*H*-pyran-3-yl)-7-(4-methylphenyl)-(*E*)-2,3,6,7-tetrahydro-1,4-thiazepine (**2a**) as an inducer of apoptosis using our proprietary cell- and caspase-based HTS assay. Through structure activity relationship (SAR) studies, 5-(4-hydroxy-6-methyl-2-oxo-2*H*-pyran-3-yl)-7-(2-methoxy-4-(methylthio)phenyl)-(*E*)-2,3,6,7-tetrahydro-1,4-thiazepine (**5d**) was identified as a potent apoptosis inducer with an EC_{50} value of 0.08 μM in T47D cells, which was >15-fold more potent than screening hit **2a**. Compound **5d** also was found to be highly active in a growth inhibition assay with a GI_{50} value of 0.05 μM in T47D cells and to function as an inhibitor of tubulin polymerization.

Apoptosis, or programmed cell death, is known to play a pivotal role in normal embryonic development, as well as for daily elimination of dispensable or excess cells.[1] Defects in apoptosis signaling pathways are hallmarks of certain cancers, resulting in uncontrollable tumor cell growth.[2] In addition, many chemotherapeutics are known to kill cancer cells through the induction of apoptosis.[3] Therefore, promoting apoptosis is a promising approach for anticancer drug discovery that could lead to the development of novel chemotherapeutic agents.[4]

We have recently reported the discovery of several series of apoptosis inducers and their molecular targets using our chemical genetics approach via application of a cell- and caspase-based HTS assay,[5] including the identification of 2-amino-3-cyano-7-dimethylamino-4-aryl-4*H*-chromene (**1a**),[6] gambogic acid (**1b**),[7] 3-aryl-5-aryl-1,2,4-oxadiazoles (**1c**),[8] and 4-anilino-2-(2-pyridyl)pyrimidines (**1d**)[9] as novel apoptosis inducers (Chart 1). Herein, we report the discovery of 5-(4-hydroxy-6-methyl-2-oxo-2*H*-pyran-3-yl)-7-(4-methylphenyl)-(*E*)-2, 3,6,7-tetrahydro-1,4-thiazepine (**2a**) as a novel apoptosis inducer using our HTS assay, and the SAR studies of **2a**, which led to the identification of **5d** as a potent inducer of apoptosis.

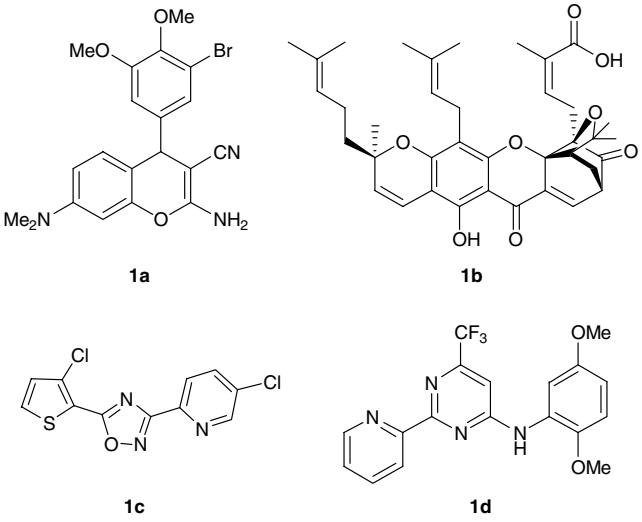

Chart 1.

Substituted 5-(4-hydroxy-6-methyl-2-oxo-2*H*-pyran-3-yl)-7-phenyl-(*E*)-2,3,6,7-tetrahydro-1,4-thiazepines

Keywords: Apoptosis inducer; HTS assay; Tubulin inhibitor.
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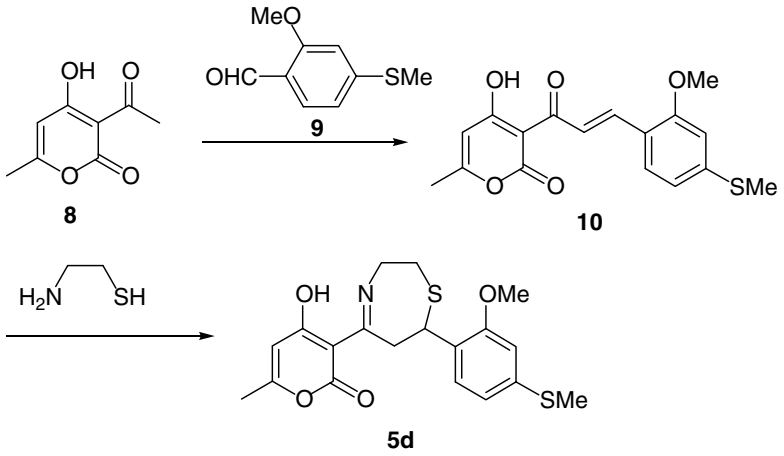


Scheme 1.

2a–**2b**, **2d**, **2g**, **3a**–**3e**, **4a**–**4d**, **5c**, and **6a**–**6b** were obtained commercially and their structures were confirmed by ^{1}H NMR and MS. Other substituted thiazepines were prepared by a two-step reaction as shown in Scheme 1 according to reported procedure.[10] For example, reaction of 3-acetyl-4-hydroxy-6-methyl-2*H*-pyran-2-one (**8**) with 2-methoxy-4-(methylthio)benzaldehyde (**9**) in ethanol and piperidine produced the substituted 4-hydroxy-6-methyl-3-((*E*)-3-(2-methoxy-4-(methylthio)phenyl)acryloyl)-2*H*-pyran-2-one (**10**), which was reacted with 2-aminoethanethiol in ethanol to produce 5-(4-hydroxy-6-methyl-2-oxo-2*H*-pyran-3-yl)-7-(2-methoxy-4-(methylthio)phenyl)-(*E*)-2,3,6,7-tetrahydro-1,4-thiazepine (**5d**).

Compound **2a** was identified as an apoptosis inducer from our cell- and caspase-based HTS assay and was characterized in detail. Treatment of T47D cells with 2 μM of **2a** for 24 h, followed by treatment with propidium iodide and analysis using flow cytometry, showed that **2a** caused an accumulation of cells with G_2/M DNA content (**2a**, 64% G_2/M vs DMSO control, 16% G_2/M), which is similar to T47D cells treated with 0.05 μM of taxol under the same conditions (taxol, 70% G_2/M) (Fig. 1). T47D cells treated with **2a** or taxol for 48 h showed 16% and 33% apoptotic subdiploid cells, respectively. When T47D cells treated with 10 μM of **2a** for 48 h were stained with a cell permeable DNA stain (Syto 16) and visualized by fluorescence microscopy, the cells showed condensed nuclei and also some fragmented nuclei, both being characteristics of apoptotic cells (Fig. 2).

Based on these characteristics, we suspected that **2a** might be a tubulin inhibitor. In a tubulin polymerization assay, compound **2a** at 20 μM was found to inhibit polymerization of tubulin completely, similar to nocodozole, a known tubulin inhibitor. Therefore compound **2a** most probably induces apoptosis through binding to tubulin and inhibition of tubulin polymerization.

For the SAR studies, the apoptosis inducing activity of substituted 5-(4-hydroxy-6-methyl-2-oxo-2*H*-pyran-3-yl)-7-phenyl-(*E*)-2,3,6,7-tetrahydro-1,4-thiazepines was measured using our cell- and caspase-based HTS assay[11]

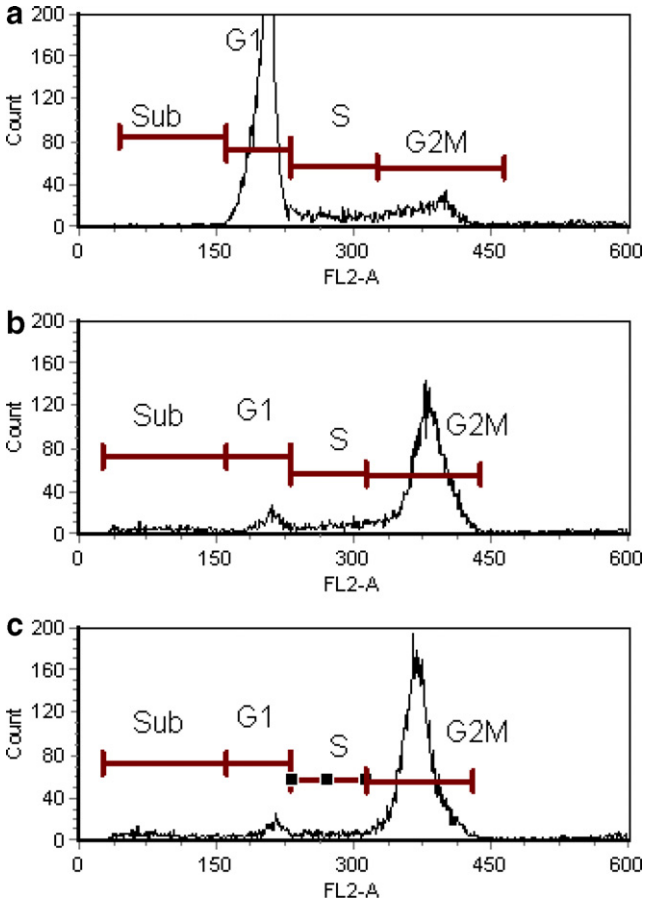


Figure 1. T47D cells were treated with compounds for 24 h and analyzed by flow cytometry. (a) Control cells treated with DMSO (65% G_1 and 16% G_2/M). (b) Cells treated with 2 μM of **2a** (7% G_1 and 64% G_2/M). (c) Cells treated with 0.05 μM of taxol (6% G_1 and 70% G_2/M).

in human breast cancer cells T47D and human colon cancer cells HCT116, and the results are summarized in Table 1. Starting from screening hit **2a**, which has a potency of 1.2 μM in T47D cells, we first explored the substituent at the *para*-position of the phenyl group. Compound **2b** was inactive in T47D cells up to 10 μM, indicating that a substituent at the 4-position is

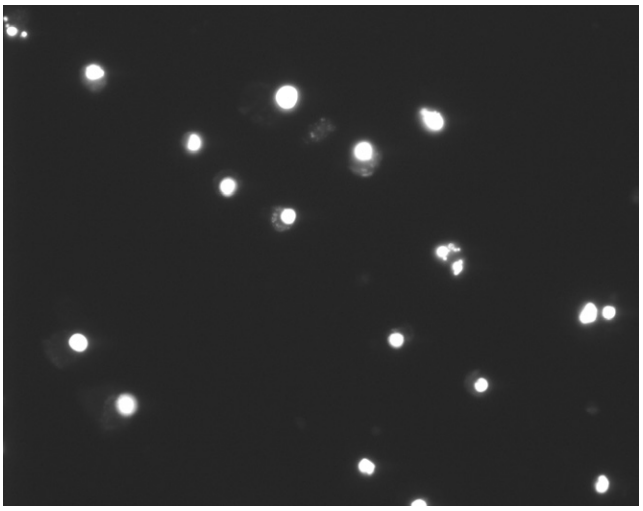
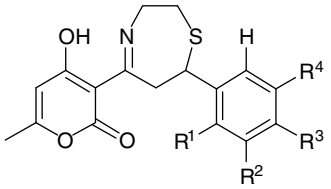


Figure 2. T47D cells were treated with 10 μM of **2a** for 48 h, stained with a cell permeable DNA stain (Syto 16), and visualized by fluorescence microscopy. Treated cells showed condensed nuclei and also some fragmented nuclei; both are characteristics seen with apoptotic cells.

important for the apoptosis inducing activity. The 4-nitro analog **2c** also was inactive up to 10 μM, suggesting that a strong electron-withdrawing group is not preferred. In comparison, the 4-Cl, 4-F, and 4-SMe analogs **2d**–**2f** were all active, suggesting that a small hydrophobic substituent at the 4-position is preferred. The 4-OH analog **2g** was inactive up to 10 μM, suggesting that a hydrophilic group is not preferred at the 4-position. Compounds **2h**–**2j** were all inactive up to 10 μM, suggesting that a large group is not preferred.

We then explored substituent effects at the *meta*- and *ortho*-positions of the phenyl group (Table 1). Among the 3-substituted analogs, compounds **3a**–**3d** were all inactive up to 10 μM. Analog **3e** (3-OMe) was the only active compound with an EC_{50} value of 4.5 μM, which was more active than the non-substituted analog **2b**. Overall, these data suggested that substitution at the 3-position is not preferred. Compounds **4a** and **4b** were both inactive up to 10 μM, indicating that similar to the 4-position, strong electron-withdrawing group and hydrophilic group are not preferred at the 2-position.

Table 1. SAR of 5-(4-hydroxy-6-methyl-2-oxo-2*H*-pyran-3-yl)-7-phenyl-(*E*)-2,3,6,7-tetrahydro-1,4-thiazepines in the caspase activation assay



Entry	R^1	R^2	R^3	R^4	EC_{50}[a] (μM)	
					T47D	HCT116
2a	H	H	Me	H	1.19 ± 0.014	1.29 ± 0.13
2b	H	H	H	H	>10	>10
2c	H	H	NO_2	H	>10	>10
2d	H	H	Cl	H	2.04 ± 0.17	2.65 ± 0.081
2e	H	H	F	H	0.30 ± 0.036	ND
2f	H	H	SMe	H	2.77 ± 0.082	2.63 ± 0.059
2g	H	H	OH	H	>10	ND
2h	H	H	OEt	H	>10	>10
2i	H	H	*i*-Pr	H	>10	>10
2j	H	H	OBz	H	>10	>10
3a	H	NO_2	H	H	>10	>10
3b	H	Br	H	H	>10	>10
3c	H	F	H	H	>10	>10
3d	H	OH	H	H	>10	>10
3e	H	OMe	H	H	4.54 ± 0.17	5.37 ± 0.10
4a	NO_2	H	H	H	>10	>10
4b	OH	H	H	H	>10	>10
4c	Br	H	H	H	2.71 ± 0.13	>10
4d	OMe	H	H	H	2.83 ± 0.19	4.099 ± 0.30
5a	OMe	H	Me	H	0.17 ± 0.017	ND
5b	OMe	H	Cl	H	0.29 ± 0.063	0.37 ± 0.050
5c	OMe	H	OMe	H	0.41 ± 0.069	0.56 ± 0.010
5d	OMe	H	SMe	H	0.077 ± 0.007	0.11 ± 0.013
5e	OMe	H	SO_2Me	H	0.13 ± 0.007	0.25 ± 0.033
5f	OMe	H	NMe_2	H	0.33 ± 0.010	0.53 ± 0.055
6a	H	OMe	OMe	H	0.57 ± 0.011	0.79 ± 0.072
6b	OMe	OMe	H	H	2.66 ± 0.20	3.69 ± 0.42
7a	OMe	OMe	OMe	H	0.17 ± 0.014	ND

ND, not determined.

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

Table 2. Comparison of caspase activation activity and growth inhibition activity of 5-(4-hydroxy-6-methyl-2-oxo-2*H*-pyran-3-yl)-7-phenyl-(*E*)-2,3,6,7-tetrahydro-1,4-thiazepines

Entry	T47D			HCT116		
	EC_{50} [a] (μM)	GI_{50} [b] (μM)	GI_{50}/EC_{50}	EC_{50} [a] (μM)	GI_{50} [b] (μM)	GI_{50}/EC_{50}
2a	1.19 ± 0.014	0.89 ± 0.21	0.8	1.29 ± 0.13	1.69 ± 0.04	1.3
5b	0.29 ± 0.063	0.30 ± 0.09	1.0	0.37 ± 0.050	0.35 ± 0.03	1.0
5c	0.41 ± 0.069	0.21 ± 0.03	0.5	0.56 ± 0.010	0.25 ± 0.05	0.4
5d	0.077 ± 0.007	0.048 ± 0.010	0.6	0.11 ± 0.013	0.095 ± 0.016	0.8
5f	0.33 ± 0.010	0.47 ± 0.08	1.4	0.53 ± 0.055	0.49 ± 0.11	0.9
6a	0.57 ± 0.011	0.51 ± 0.03	0.9	0.79 ± 0.072	0.53 ± 0.03	0.7

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

Compounds **4c** and **4d** were both active, with potencies in the 2 μM range, suggesting that a small substituent such as OMe at the 2-position contributes positively to the apoptosis inducing activity.

Since the SAR data show that a substituent at the 4-position is important for activity, and a small group at the 2-position contributes positively to activity, we explored the combination of substituents at the 4-position with an OMe group at the 2-position. Compounds **5a**–**5f** were all more active than the corresponding 4-substituted and 2-OMe substituted analogs. Compound **5d** was the most potent analog, with an EC_{50} value of 0.08 μM. In comparison, the 3,4-diOMe analog **6a** and 2,3-dimethoxy analog **6b** were less active than the 2,4-diOMe analog **5c**. The 2,3,4-trimethoxy analog **7a** was more potent than 2,4- and 3,4-diOMe analogs **5c** and **6a**, confirming that an OMe group at the 2- and 3-position contributes positively to activity.

Overall, the trend of apoptosis inducing activities of the substituted thiazepines in human breast cancer T47D cells was similar to that observed in human colon cancer HCT116 cells (Table 1). All of the compounds that were active in T47D cells were active in HCT116 cells, and all of the compounds that were inactive in T47D cells up to 10 μM also were found to be inactive in HCT116 cells. Compound **5d**, the most active analog in T47D cells, also was the most active one in HCT116 with an EC_{50} value of 0.1 μM.

Selected thiazepines were assayed in a traditional growth inhibition (GI_{50}) assay to confirm that those active in the caspase induction assay also inhibit tumor cell growth. The growth inhibition assays in T47D and HCT116 cells were run in a 96-well microtiter plate as described previously[9] and the data are summarized in Table 2. Compound **5d** had a GI_{50} value of 0.05 and 0.1 μM in T47D and HCT116 cells, respectively. In general, the compounds more active in the apoptosis induction assay, as measured by caspase activation, also are more potent in the growth inhibition assay, and the ratio of GI_{50}/EC_{50} is around 1 for all the tested compounds. Compound **5d** also was tested in the tubulin polymerization assay and was found to be more active than hit **2a,** inhibiting tubulin polymerization completely at 5 μM. Similar to what we reported earlier,[6,11] these data confirm that the cell-based caspase activation HTS assay is not only useful for the identification of inducers of apoptosis, but also is useful for subsequent optimization and SAR study of screening hits.

In conclusion, we have identified a series of substituted 5-(4-hydroxy-6-methyl-2-oxo-2*H*-pyran-3-yl)-7-phenyl-(*E*)-2,3,6,7-tetrahydro-1,4-thiazepines as potent apoptosis inducers. Compound **2a** was found to arrest cancer cells in G_2/M and to inhibit tubulin polymerization, which most probably is its mechanism of action for induction of apoptosis. Through SAR studies, compound **5d** was identified as the most potent compound in this series with an EC_{50} value of 0.08 μM in T47D cells, which was >15-fold more potent than screening hit **2a**. Compound **5d** also is highly active in the growth inhibition assay with a GI_{50} value of 0.05 μM in T47D cells and functions by inhibiting tubulin polymerization.

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