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Synthesis, antiproliferative, and antiplatelet activities of oxime- and methyloxime-containing flavone and isoflavone derivatives

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Abstract—Certain oxime- and methyloxime-containing flavone and isoflavone derivatives were synthesized and evaluated for their antiproliferative activity against three solid cancer cells, human cervical epithelioid carcinoma (HeLa), hepatocellular carcinoma (SKHep1), and oral squamous cell carcinoma (SAS), which are commonly seen in Asian countries, including Taiwan. Selective compounds were also evaluated in the full panel of 60 human tumor cell lines and their mean GI_{50} values were obtained. The preliminary assays indicated flavone-6-yl derivatives are the most cytotoxic while isoflavone-7-yl derivatives are the best antiplatelet agents. Among them, (*E*)-6-(2-methoxyiminopropoxy)-2-phenyl-4*H*-1-benzopyran-4-one (**14**), (*Z*)-6-(2-hydroxyimino-2-phenylethoxy)-2-phenyl-4*H*-1-benzopyran-4-one (**18a**), and (*Z*)-6-[2-hydroxyimino-2-(4-methoxyphenyl)ethoxy]-2-phenyl-4*H*-1-benzopyran-4-one (**18c**) are three of the best antiproliferative agents with GI_{50} values of 0.8, 0.7, and 0.8 μM, respectively, against the growth of SKHep1; 0.9, 0.8, and 1.0 μM, respectively, against the growth of HeLa cells. Compound **18c** is not only the most cytotoxic with a mean GI_{50} value of 0.08 μM against the full panel of 60 human tumor cell lines but also the only flavone derivative that exhibited a GI_{50} value of less than 1 μM against the growth of SAS. Flow cytometric analyses revealed that growth inhibition by **18c** was due to accumulation in G2/M phase arrest and followed by apoptosis.

1. Introduction

Flavonoids and isoflavonoids are ubiquitous families of natural products that possess a wide variety of biological activities, including antiproliferative,[1–8] antifungal,[9] antiviral,[10] anti-inflammatory,[11] antioxidant,[12,13] and cardiovascular effects.[14–18] Recently, we have reported preparation of certain flavone and isoflavone derivatives and investigated their antiproliferative activity in a detailed structure–activity relationship (SAR) study.[19–21] The antiproliferative assay was evaluated in the full panel of 60 human tumor cell lines derived from nine cancer cell types (leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, and breast cancer). Since human cervical epithelioid carcinoma (HeLa), hepatocellular carcinoma (SKHep1), and oral squamous cell carcinoma (SAS) are commonly seen in Asian countries including Taiwan, the present report describes the preparation of certain oxime- and methyloxime-containing flavone and isoflavone, and their antiproliferative evaluation against these three solid cancers. Selective compounds were also evaluated in the full panel of 60 human tumor cell lines and their mean GI_{50} values were obtained.

A number of flavone and isoflavone derivatives have been found to exhibit antiplatelet and vasorelaxing activities.[15–18] This prompted us to investigate the antiplatelet effect of these flavone and isoflavone oximes in an attempt to identify potential drug candidates which selectively inhibit either the platelet aggregation or the growth of cancer cells.

Keywords: Antiproliferative activity; Antiplatelet activity; Cytotoxicity; Apoptosis; Flavone; Isoflavone.
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2. Chemistry

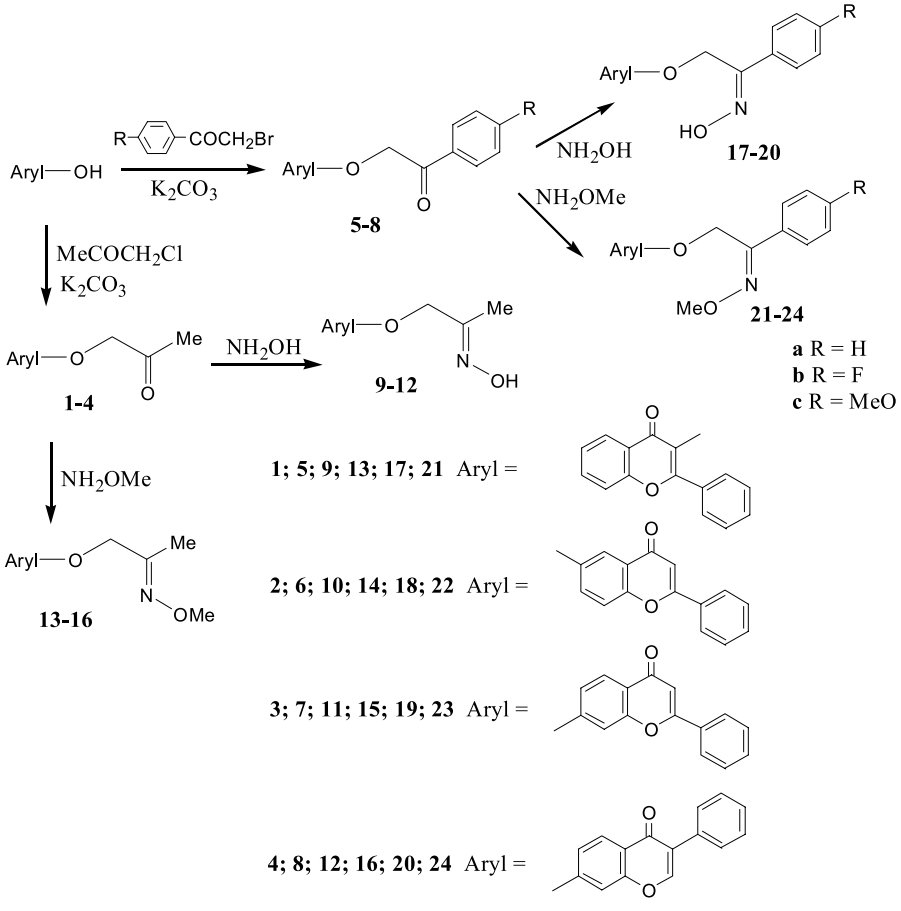
The preparation of oxime- and methyloxime-containing flavone and isoflavone derivatives is illustrated in Scheme 1. Alkylation of 3-hydroxyflavone with chloroacetone under basic conditions gave 3-(2-oxopropoxy)-2-phenyl-4*H*-1-benzopyran-4-one (**1**),[18] which was then treated with NH_2OH to afford exclusively (*E*)-3-(2-hydroxyiminopropoxy)-2-phenyl-4*H*-1-benzopyran-4-one (**9**) in a good overall yield. The configuration of the oxime moiety was determined by through-space nuclear Overhauser effect spectroscopy (NOESY), which revealed coupling connectivity to CH_3 protons. Accordingly, reaction of 3-hydroxyflavone with bromomethyl ketones gave their respective phenylketone derivatives **5a**–**c**[18] which were treated with NH_2OH to give (*Z*)-**17a**–**c**. The same synthetic procedure was applied for the synthesis of flavone-6-yl oximes, (*E*)-**10** from 6-(2-oxopropoxy)-2-phenyl-4*H*-1-benzopyran-4-one (**2**);[18] (*Z*)-**18a**–**c** from **6a**–**c**,[18] respectively; and flavone-7-yl oximes (*E*)-**11**[21] from 7-(2-oxopropoxy)-2-phenyl-4*H*-1-benzopyran-4-one (**3**);[16] (*Z*)-**19a**–**c**[21] from **7a**–**c**,[16] respectively; and isoflavone-7-yl oximes, (*E*)-**12**[21] from 7-(2-oxopropoxy)-3-phenyl-4*H*-1-benzopyran-4-one (**4**); (*Z*)-**20a**–**c**[21] from **8a**–**c**, respectively. The configuration of the oxime moiety was further confirmed by the ^{13}C NMR spectra. The carbon of 1′-CH_2 which is *anti* to the oxime moiety shifted downfield (δ 73.14 for (*E*)-9 and 70.00 ppm for (*E*)-**10**), while that of the *syn* isomer shifted upfield (δ 61.90 for (*Z*)-**17a**, 61.82 for (*Z*)-**17b**, 61.85 for (*Z*)-**17c**, 59.38 for (*Z*)-**18a**, 59.36 for (*Z*)-**18b**, and 59.30 ppm for (*Z*)-**18c**).[22]

Reaction of **1** and **5a**–**c** with NH_2OMe provided (*E*)-3-(2-methoxyiminopropoxy)-2-phenyl-4*H*-1-benzopyran-4-one (**13**) and (*Z*)-**21a**–**c**, respectively. The same synthetic procedure was applied for the synthesis of flavone-6-yl oxime methylethers, (*E*)-**14** from **2**; (*Z*)-**22a**–**c** from **6a**–**c**, respectively; and flavone-7-yl oxime methylethers, (*E*)-**15**[21] from **3**; (*Z*)-**23a**–**c**[21] from **7a**–**c**, respectively; and isoflavone-7-yl oxime methylethers, (*E*)-**16**[21] from **4**; (*Z*)-**24a**–**c**[21] from **8a**–**c**, respectively.

3. Pharmacological results and discussion

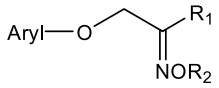
3.1. Antiproliferative activity

All compounds were evaluated in vitro against a three-cell line panel consisting of human cervical epithelioid carcinoma HeLa, hepatocellular carcinoma SKHep1, and oral squamous cell carcinoma SAS. Results from Table 1 indicated the optimal hydrophilicity is crucial for the antiproliferative activity of flavone-6-yl derivatives. When R_1 is a less hydrophobic methyl group, R_2 prefers to be a methoxy rather than a polar hydroxyl



Scheme 1.

Table 1. Antiproliferative activity of flavone and isoflavone derivatives



Compound	Substituents			GI_{50} (μM)[a]			Mean GI_{50} (μM)[b,c]
	Aryl	R_1	R_2	SKHep1	HeLa	SAS	
9	Flavone-3-yl	Me	H	2.6 ± 0.04	2.0 ± 0.23	5.8 ± 1.90	nd
10	Flavone-6-yl	Me	H	1.9 ± 0.24	1.2 ± 0.18	.9 ± 0.98	nd
11	Flavone-7-yl	Me	H	2.8 ± 0.33	2.0 ± 1.34	2.5 ± 1.17	nd
12	Isoflavone-7-yl	Me	H	7.3 ± 0.96	9.8 ± 1.23	6.3 ± 1.32	nd
13	Flavone-3-yl	Me	Me	9.2 ± 1.03	2.0 ± 0.82	3.4 ± 0.21	nd
14	Flavone-6-yl	Me	Me	0.8 ± 0.75	0.9 ± 0.21	2.0 ± 0.71	nd
15	Flavone-7-yl	Me	Me	2.0 ± 0.12	2.2 ± 0.15	3.2 ± 0.47	nd
16	Isoflavone-7-yl	Me	Me	6.4 ± 1.09	8.5 ± 1.28	2.6 ± 0.71	nd
17a	Flavone-3-yl	Ph	H	7.2 ± 0.58	1.8 ± 0.20	7.5 ± 1.32	13.2
17b	Flavone-3-yl	4-F-Ph	H	6.9 ± 0.19	2.1 ± 0.03	5.9 ± 1.15	nd
17c	Flavone-3-yl	4-MeO-Ph	H	2.7 ± 0.14	2.0 ± 0.02	7.7 ± 1.66	12.3
18a	Flavone-6-yl	Ph	H	0.7 ± 0.21	0.8 ± 0.21	2.6 ± 0.02	3.7
18b	Flavone-6-yl	4-F-Ph	H	2.8 ± 1.27	1.6 ± 0.34	2.4 ± 0.48	4.5
18c	Flavone-6-yl	4-MeO-Ph	H	0.8 ± 0.25	1.0 ± 1.12	0.8 ± 0.03	0.08
19a	Flavone-7-yl	Ph	H	2.0 ± 0.15	2.0 ± 0.36	6.0 ± 0.47	19.5
19b	Flavone-7-yl	4-F-Ph	H	2.3 ± 0.92	2.0 ± 0.17	2.6 ± 0.20	21.4
19c	Flavone-7-yl	4-MeO-Ph	H	5.6 ± 0.68	2.0 ± 0.14	2.0 ± 0.16	12.3
20a	Isoflavone-7-yl	Ph	H	6.4 ± 1.27	9.0 ± 1.09	3.0 ± 0.95	16.5
20b	Isoflavone-7-yl	4-F-Ph	H	6.6 ± 1.17	7.8 ± 1.10	2.9 ± 0.53	16.2
20c	Isoflavone-7-yl	4-MeO-Ph	H	5.5 ± 1.21	7.6 ± 0.87	5.6 ± 1.05	2.84
21a	Flavone-3-yl	Ph	Me	7.1 ± 0.43	1.6 ± 1.06	2.0 ± 0.16	nd
21b	Flavone-3-yl	4-F-Ph	Me	13 ± 1.21	2.0 ± 0.81	3.7 ± 1.12	nd
21c	Flavone-3-yl	4-MeO-Ph	Me	11 ± 0.68	2.0 ± 0.48	2.3 ± 0.28	nd
22a	Flavone-6-yl	Ph	Me	8.2 ± 0.53	2.4 ± 0.25	6.0 ± 1.33	nd
22b	Flavone-6-yl	4-F-Ph	Me	7.9 ± 1.24	2.3 ± 0.14	2.7 ± 0.18	nd
22c	Flavone-6-yl	4-MeO-Ph	Me	2.0 ± 0.15	2.0 ± 0.26	5.0 ± 1.21	nd
23a	Flavone-7-yl	Ph	Me	2.0 ± 0.08	6.6 ± 0.68	2.0 ± 0.04	nd
23b	Flavone-7-yl	4-F-Ph	Me	3.3 ± 1.11	2.7 ± 0.31	4.1 ± 1.15	nd
23c	Flavone-7-yl	4-MeO-Ph	Me	4.8 ± 1.24	2.5 ± 0.14	2.7 ± 0.20	nd
24a	Isoflavone-7-yl	Ph	Me	7.4 ± 0.81	8.2 ± 1.30	3.9 ± 0.61	nd
24b	Isoflavone-7-yl	4-F-Ph	Me	6.1 ± 1.48	6.4 ± 1.22	4.9 ± 0.78	nd
24c	Isoflavone-7-yl	4-MeO-Ph	Me	5.3 ± 1.05	7.3 ± 0.26	2.5 ± 0.45	nd

[a] GI_{50}: drug molar concentration causing 50% cell growth inhibition ($n = 3$).

[b] Data obtained from NCI's in vitro disease-oriented tumor cell screen.

[c] Mean GI_{50}: mean values over all cell lines tested. Theses cell lines are: leukemia (CCRF-CEM, HL-60 (TB), K-562, MOLT-4, PRMI-8226, and SR); non-small cell lung cancer (A549/ATCC, EKVX, HOP-62, HOP-92, NCI-H226, NCI-H23, NCI-H322M, NCI-H460, and NCI-H522); colon cancer (COLC 205, HCC-2998, HCT-116, HCT-15, HT29, KM12, and SW-620); CNS cancer (SF-268, SF-295, SF-539, SNB-19, SNB-75, and U251); melanoma (LOX IMVI, MALME-3M, M14, SK-MEL-2, SK-MEL-28, SK-MEL-5, and UACC-257); ovarian cancer (IGROV1, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, and SK-OV-3); renal cancer (786-0, A498, ACHN, CAKI-1, RXF 393, SN12C, TK-10, and UO-31); prostate cancer (PC-3 and DU-145) and breast cancer (MCF7, MCF7/ADR-RES, MDA-MB-231/ATCC, HS 578T, MDA-MB-435, MDA-N, and T-47D).

group (**14** vs. **10**). However, when R_1 is a more hydrophobic phenyl or a substituted phenyl group, R_2 preferred to be a polar hydroxyl rather than a methoxy group (**18a**–**c** vs. **22a**–**c**). For the (2-hydroxyiminopropoxy) derivatives, aryl group is preferred to be flavone-6-yl as **10** exhibited the most strong antiproliferative activity among its isomers (**10** vs. **9**, **11**, and **12**). The same trend was observed for its methyl ether counterparts in which flavone-6-yl **14** is the most potent in comparison to **13**, **15**, and **16**. Accordingly, (2-hydroxyimino-2-phenylethoxy) group substituted at the C-6 position of flavone skeleton is the most active (**18a** vs. **17a**, **19a**, and **20a**). For the flavone-6-yl derivatives, **14**, **18a**, and **18c** are three of the best with GI_{50} values of 0.8, 0.7, and 0.8 μM, respectively, against hepatocellular carcinoma (SKHep1); 0.9, 0.8, and 1.0 μM, respectively, against the cervical epithelioid carcinoma (HeLa). (*Z*)-6-[2-Hydroxyimino-2-(4-methoxyphenyl)ethoxy]-2-phenyl-4*H*-1-benzopyran-4-one (**18c**) is the only compound which exhibited a GI_{50} value of less than 1 μM ($GI_{50} = 0.8$ μM) against oral squamous cell carcinoma (SAS).

Selective compounds were evaluated in the full panel of 60 human tumor cell lines derived from nine cancer cell types (leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, and breast cancer) and the mean GI_{50} values were calculated.[23] An electron-donating 4-methoxyphenyl group at R_1 of flavone-6-yl derivatives is more active than the phenyl or the 4-fluorophenyl substituent (**18c**, mean $GI_{50} = 0.08$ μM; **18a**, 3.7 μM; **18b**,

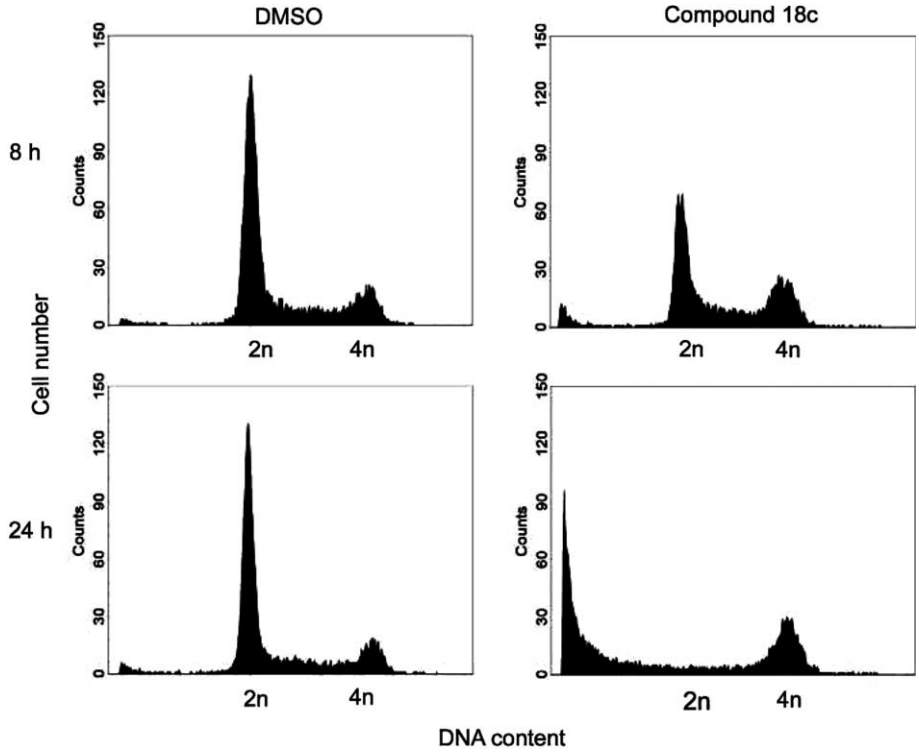


Figure 1. Effects of **18c** on cell cycle in HeLa cells. After treatment with DMSO or 5 μM of **18c** for 8 and 24 h, the cells were detached from substratum by trypsinization, washed by PBS, and stained with PI followed by Becton–Dickinson FACScan flow cytometer analysis.

4.5 μM). The same antiproliferative SAR was observed for flavone-7-yl (**19c**, mean GI_{50} = 12.3 μM; **19a**, 19.5 μM; **19b**, 21.4 μM) and isoflavone-7-yl (**20c**, mean GI_{50} = 2.84 μM; **20a**, 16.5 μM; **20b**, 16.2 μM) derivatives. The results of this study showed that the antiproliferative activity decreased in the order of linked chromophore flavone-6-yl **18a**–**c** > isoflavone-7-yl **20a**–**c** > flavone-3-yl **17a**–**c** and flavone-7-yl **19a**–**c**. Among them, **18c** was the most cytotoxic with a mean GI_{50} value of 0.08 μM and therefore, was further evaluated on its effect of cell cycle distribution and apoptosis as illustrated in Figure 1. The peak before the G1 phase on histogram is called apoptotic cells. The proportion of cells was slightly increased in the sub-G1 and accumulated in G2/M phase, however, was decreased in the G0/G1 phase of the cell cycle after 8 h treatment. After 24 h, the accumulation of the cells in G0/G1 DNA content was significantly decreased while the hypodiploid (sub-G0/G1 phase) cells increased. Compound **18c** inhibited proliferation of HeLa by the alteration of cell division, accumulation of cells in G2/M phase at early 8 h which was then decreased followed by the increase of apoptotic cells (sub-G1 phase) after 24 h treatment. Thus, compound **18c** induces cell cycle arrest followed by apoptosis.

3.2. Antiplatelet activity

The antiplatelet activities were evaluated in washed rabbit platelets. Platelet aggregation was induced by thrombin (Thr, 0.1 U/ml), arachidonic acid (AA, 200 μM), and collagen (Col, 10 μg/ml), respectively. The final concentration of compounds was 100 μM and the results are shown in Table 2. All of them were found to be inactive against Thr-induced aggregation and only marginally active or inactive against Col-induced aggregation at 100 μM. However, most of them were capable of inhibiting the platelet aggregation perfectly which was induced by AA at the same concentration. For 2-hydroxyiminopropoxy derivatives, the potency decreased in the order of linked chromophore isoflavone-7-yl (**12**, IC_{50} = 2.97 μM) > flavone-7-yl (**11**, IC_{50} = 7.7 μM) > flavone-3-yl (**9**, IC_{50} = 38.8 μM) > flavone-6-yl (**10**, inactive). With exception of **18a** (IC_{50} = 25.9 μM), the same antiplatelet SAR was observed for 2-hydroxyimino-2-(4-substituted)phenylethoxy derivatives, in which the potency decreased in the order of isoflavone-7-yl **20a**–**c** > flavone-7-yl **19a**–**c** > flavone-3-yl **17a**–**c** > flavone-6-yl **18b** and **18c**; and 2-methoxyiminopropoxy derivatives in which isoflavone-7-yl (**16**, IC_{50} = 13.6 μM) is active while its isomers **13**–**15** are inactive. With exception of **23a** (IC_{50} = 14.5 μM), 2-methoxyimino-2-(4-substituted)phenylethoxy derivatives of flavone-3-yl **21a**–**c**, flavone-6-yl **22a**–**c**, and flavone-7-yl **23b** and **23c** are inactive. For 2-hydroxyimino-2-(4-substituted)phenylethoxy derivatives of flavone-3-yl **17a**–**c**, the potency of **17a**, **17b**, and **17c** is comparable with IC_{50} of 42.6, 40.7, and 30.2 μM, respectively. Comparable antiplatelet SAR was also observed for the flavone-7-yl counterparts **19a**–**c**.

4. Conclusion

A number of oxime- and methyloxime-containing flavone and isoflavone derivatives were synthesized and

Table 2. Effects of flavone and isoflavone derivatives on the platelet aggregation

Compounds (100 μM)		Thrombin (0.1 U/ml)	Arachidonic acid (200 μM)	Collagen (10 μg/ml)
Control		91.2 ± 1.1	88.1 ± 1.7	90.8 ± 0.7
9		90.3 ± 0.5	0[c]	9.2 ± 7.5[c]
	IC_{50}		38.8 ± 1.8	54.4 ± 4.6
10		89.4 ± 0.5	85.4 ± 2.4	92.1 ± 0.9
11		88.6 ± 1.4	0[c]	18.2 ± 8.2[c]
	IC_{50}		7.7 ± 1.6	48.3 ± 7.9
12		88.3 ± 0.4	0[c]	32.5 ± 1.7[c]
	IC_{50}		2.97 ± 0.4	36.8 ± 10.3
13		83.6 ± 1.3	0[c]	8.81 ± 7.2[c]
	IC_{50}		74.5 ± 0.4	72.7 ± 4.3
14		86.8 ± 1.1	83.7 ± 1.7	84.9 ± 3.4
15		86.2 ± 1.7	7.94 ± 3.8[c]	16.2 ± 0.4
	IC_{50}		76.5 ± 1.2	80.8 ± 0.1
16		85.7 ± 0.5	0[c]	14.0 ± 4.2[c]
	IC_{50}		13.6 ± 7.9	33.2 ± 5.6
17a		88.6 ± 1.1	0[c]	26.5 ± 2.8[c]
	IC_{50}		42.6 ± 0.8	38.1 ± 5.5
17b		86.6 ± 1.0	0[c]	83.9 ± 1.7
	IC_{50}		40.7 ± 0.5	
17c		90.5 ± 0.6	0[c]	41.4 ± 5.8
	IC_{50}		30.2 ± 9.3	45.8 ± 17.8
18a		88.1 ± 0.8	0[c]	85.2 ± 4.8
	IC_{50}		25.9 ± 6.6	
18b		86.9 ± 0.7	85.7 ± 4.7	92.0 ± 0.3
18c		87.6 ± 1.7	87.9 ± 1.4	56.6 ± 6.4
19a		88.3 ± 1.7	0[c]	12.8 ± 1.3[c]
	IC_{50}		29.4 ± 3.9	46.4 ± 2.4
19b		86.3 ± 1.9[a]	0[c]	14.7 ± 0.8[c]
	IC_{50}		36.7 ± 1.4	43.9 ± 6.2
19c		85.2 ± 1.4[a]	0[c]	20.3 ± 4.1[c]
	IC_{50}		29.0 ± 3.3	55.3 ± 4.9
20a		83.9 ± 1.9	0[c]	23.4 ± 4.8[c]
	IC_{50}		11.6 ± 1.9	28.9 ± 1.1
20b		88.3 ± 1	0[c]	23.1 ± 2[c]
	IC_{50}		8.0 ± 0.4	34.7 ± 3.3
20c		83.6 ± 0.9	0[c]	22.3 ± 2.8[c]
	IC_{50}		11.6 ± 1.7	28.5 ± 7.2
21a		86.6 ± 0.08	82.3 ± 2.2	66.5 ± 6.7[b]
21b		83.1 ± 0.5	82.6 ± 2.2	72.2 ± 4.8[b]
21c		84.6 ± 1.3	83.9 ± 0.9	47.9 ± 6.9[c]
22a		87.6 ± 0.66	84.1 ± 0.7	83.1 ± 0.8
22b		85.7 ± 1.2	81.6 ± 2.2	69.6 ± 8.8
22c		84.3 ± 0.92	79.1 ± 3.0	81.0 ± 0.01
23a		85.1 ± 0.35	0[c]	10.79 ± 0
	IC_{50}		14.50 ± 0.97	65.31 ± 0
23b		85.6 ± 1.1	79.10 ± 3.1	60.91 ± 0
23c		82.5 ± 0.93	78.60 ± 1.1[c]	79.62 ± 0
24a		74.5 ± 2.8	0[c]	13.8 ± 6.5[c]
	IC_{50}		20.8 ± 4.8	54.1 ± 13.7
24b		79.7 ± 2.7	0[c]	16.4 ± 1.9[c]
	IC_{50}		43.7 ± 8.3	49.0 ± 8.6
24c		71.8 ± 6.6	89 ± 1.6	47.7 ± 17.3

[a] Significantly different from control value at $P < 0.05$.
[b] Significantly different from control value at $P < 0.01$.
[c] Significantly different from control value at $P < 0.001$.

evaluated for their antiplatelet and antiproliferative activities. The results indicated flavone-6-yl derivatives are most cytotoxic while isoflavone-7-yl derivatives are best antiplatelet agents. Our findings that **14** and **18c** being inactive against the platelet aggregation are interesting, because both compounds were found to be potent antiproliferative agents against the growth of three solid cancer cells, HeLa, SKHep1, and SAS. Exposure to **18c** had a strong antiproliferative effect on HeLa cells and caused an increase in the population of apoptotic cells. A significant number of cells were accumulated in G2/M phase. Thus, compound **18c** induces cell cycle arrest followed by apoptosis.

5. Experimental

5.1. General

TLC: precoated (0.2 mm) silica gel 60 F_{254} plates from EM Laboratories, Inc.; detection by UV light (254 nm). mp: *Electrothermal IA9100* digital melting-point apparatus; uncorrected. ^{1}H NMR spectra: Varian-Unity-400 spectrometer at 400 or Varian-Gemini-200 spectrometer at 200, chemical shifts δ in ppm with $SiMe_4$ as an internal standard (=0 ppm), coupling constants J in hertz. Elemental analyses were carried out on a Heraeus CHN-O-Rapid elemental analyzer, and results were within ±0.4% of calculated values.

5.1.1. (*E*)-3-(2-Hydroxyiminopropoxy)-2-phenyl-4*H*-1-benzopyran-4-one (9). To a solution of **1**[18] (0.29 g, 1 mmol) in EtOH (20 ml) was added a solution of hydroxylamine hydrochloride (0.14 g, 2 mmol) in EtOH (2 ml). The mixture was heated at reflux for 24 h (TLC monitoring) and evaporated to give a residual solid. The white solid thus obtained was collected and purified by flash column chromatography (FC; silica gel; CH_2Cl_2/EtOAc 4:1) and recrystallized from CH_2Cl_2 to give **9** (0.22 g, 71%). mp 144–145 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 1.60 (s, Me), 4.55 (s, OCH_2), 7.48–7.52 (m, 1H, arom. H), 7.55–7.63 (m, 3H, arom. H), 7.73–7.89 (m, 2H, arom. H), 7.97–8.06 (m, 2H, arom. H), 8.10–8.15 (m, 1H, arom. H), 10.86 (s, NOH). ^{13}C NMR (100 MHz, DMSO-d_6): 11.62 (Me), 73.14 (CH_2O), 118.38, 123.42, 124.91, 125.09, 128.43, 128.61, 130.29, 130.87, 134.09, 138.84, 151.75, 154.71, 155.88 (arom. C and C=N), 173.63 (C(4)). Anal. Calcd for $C_{18}H_{15}NO_4$: C 69.89, H 4.89, N 4.53. Found: C 69.80, H 4.92, N 4.55.

The same procedure was applied to convert **2** to **10**; **5a–c** to **17a–c**; **6a–c** to **18a–c**, respectively.

5.1.2. (*E*)-6-(2-Hydroxyiminopropoxy)-2-phenyl-4*H*-1-benzopyran-4-one (10). Yield: 81%. mp 209–210 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 1.85 (s, Me), 4.69 (s, OCH_2), 7.02 (s, 1H-C(3)), 7.47 (dd, $J = 8.8$, 3.2, 1H-C(7)), 7.50 (d, $J = 3.2$, 1H-C(5)), 7.55–7.61 (m, 3H, arom. H), 7.76 (d, $J = 8.8$, 1H-C(8)), 8.08–8.11 (m, 2H, arom. H), 11.05 (s, NOH). ^{13}C NMR (100 MHz, DMSO-d_6): 11.51 (Me), 70.00 (CH_2O), 106.18, 106.30, 120.24, 123.84, 124.03, 126.34, 129.17, 131.21, 131.81, 150.61, 151.63, 155.48, 162.40 (arom. C and C=N), 176.87 (C(4)). Anal. Calcd for $C_{18}H_{15}NO_4$: C 69.89, H 4.89, N 4.53. Found: C 69.54, H 4.89, N 4.44.

5.1.3. (*Z*)-3-(2-Hydroxyimino-2-phenylethoxy)-2-phenyl-4*H*-1-benzopyran-4-one (17a). Yield: 61%. mp 158–159 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 5.30

(s, OCH_2), 7.32–7.57 (m, 7H, arom. H), 7.74–7.89 (m, 4H, arom. H), 7.94–7.98 (m, 2H, arom. H), 8.15–8.18 (m, 1H, arom. H), 11.66 (s, NOH). ^{13}C NMR (100 MHz, DMSO-d_6): 61.90 (CH_2O), 118.40, 123.41, 125.00, 125.11, 126.32, 128.08, 128.23, 128.49, 128.63, 130.07, 130.74, 134.12, 134.65, 139.54, 152.38, 154.71, 155.38 (arom. C and C=N), 173.80 (C(4)). Anal. Calcd for $C_{23}H_{17}NO_4$: C 74.38, H 4.61, N 3.77. Found: C 74.36, H 4.64, N 3.80.

5.1.4. (*Z*)-3-[2-(4-Fluorophenyl)-2-hydroxyiminoethoxy]-2-phenyl-4*H*-1-benzopyran-4-one (17b). Yield: 71%. mp 179–180 °C. ^1H NMR (400 MHz, DMSO-d_6): 5.29 (s, OCH_2), 7.09–7.18 (m, 2H, arom. H), 7.36–7.57 (m, 4H, arom. H), 7.74–7.96 (m, 6H, arom. H), 8.14–8.18 (m, 1H, arom. H), 11.66 (s, NOH). ^{13}C NMR (100 MHz, DMSO-d_6): 61.82 (CH_2O), 114.75, 115.19, 118.40, 123.39, 124.99, 125.12, 128.22, 128.36, 128.49, 130.06, 130.71, 131.09, 134.14, 139.43, 151.56, 154.73, 155.47, 159.92, 164.80 (arom. C and C=N), 173.97 (C(4)). Anal. Calcd for $C_{23}H_{16}FNO_4$: C 70.95, H 4.14, N 3.60. Found: C 70.64, H 4.24, N 3.66.

5.1.5. (*Z*)-3-[2-Hydroxyimino-2-(4-methoxyphenyl)ethoxy]-2-phenyl-4*H*-1-benzopyran-4-one (17c). Yield: 60%. mp 156–157 °C. ^1H NMR (400 MHz, DMSO-d_6): 3.77 (s, MeO), 5.27 (s, OCH_2), 6.84–6.92 (m, 2H, arom. H), 7.37–7.57 (m, 4H, arom. H), 7.67–7.90 (m, 4H, arom. H), 7.95–8.00 (m, 2H, arom. H), 8.14–8.18 (m, 1H, arom. H), 11.44 (s, NOH). ^{13}C NMR (100 MHz, DMSO-d_6): 55.07 (MeO), 61.85 (CH_2O), 113.53, 118.37, 123.39, 124.91, 125.08, 127.08, 127.67, 128.20, 128.48, 130.09, 130.69, 134.09, 139.54, 151.90, 154.70, 155.33, 159.64 (arom. C and C=N), 173.99 (C(4)). Anal. Calcd for $C_{24}H_{19}NO_5$: C 71.81, H 4.77, N 3.49. Found: C 71.55, H 4.77, N 3.51.

5.1.6. (*Z*)-6-(2-Hydroxyimino-2-phenylethoxy)-2-phenyl-4*H*-1-benzopyran-4-one (18a). Yield: 66%. mp 212–213 °C. ^1H NMR (400 MHz, DMSO-d_6): 5.39 (s, OCH_2), 7.02 (s, 1H-C(3)), 7.35 (dd, $J = 9.2$, 3.2, 1H-C(7)), 7.37–7.42 (m, 3H, arom. H), 7.54 (d, $J = 3.2$, 1H-C(5)), 7.57–7.68 (m, 5H, arom. H), 7.71 (d, $J = 9.2$, 1H-C(8)), 8.07–8.09 (m, 2H, arom. H), 12.02 (s, NOH). ^{13}C NMR (100 MHz, DMSO-d_6): 59.38 (CH_2O), 105.84, 106.21, 120.27, 123.53, 126.33, 126.44, 128.08, 128.35, 128.57, 128.96, 129.15, 131.20, 131.80, 150.66, 152.54, 155.19, 162.37 (arom. C and C=N), 176.83 (C(4)). Anal. Calcd for $C_{23}H_{17}NO_4$: C 74.38, H 4.61, N 3.77. Found: C 74.35, H 4.59, N 3.81.

5.1.7. (*Z*)-6-[2-(4-Fluorophenyl)-2-hydroxyiminoethoxy]-2-phenyl-4*H*-1-benzopyran-4-one (18b). Yield: 75%. mp 178–179 °C. ^1H NMR (400 MHz, DMSO-d_6): 5.39 (s, OCH_2), 7.01 (s, 1H-C(3)), 7.19–7.26 (m, 2H, arom. H), 7.35 (dd, $J = 9.2$, 3.2, 1H-C(7)), 7.54 (d, $J = 3.2$, 1H-C(5)), 7.55–7.59 (m, 3H, arom. H), 7.69–7.72 (m, 2H, arom. H); 7.72 (d, $J = 9.2$, 1H-C(8)), 8.06–8.09 (m, 2H, arom. H), 12.04 (s, NOH). ^{13}C NMR (100 MHz, DMSO-d_6): 59.36 (CH_2O), 105.85, 106.18, 115.13, 115.35, 120.14, 120.22, 123.43, 124.04, 126.27, 128.56, 128.64, 129.08, 130.50, 131.72, 150.62, 151.72, 155.02, 161.24, 162.31, 163.69 (arom. C and C=N), 176.73 (C(4)). Anal. Calcd for $C_{23}H_{16}FNO_4$: C 70.95, H 4.14, N 3.60. Found: C 70.73, H 4.28, N 3.63.

5.1.8. (*Z*)-6-[2-Hydroxyimino-2-(4-methoxyphenyl)ethoxy]-2-phenyl-4*H*-1-benzopyran-4-one (18c). Yield: 90%. mp 166–167 °C. ^1H NMR (400 MHz, DMSO-d_6): 3.75 (s, MeO), 5.36 (s, OCH_2), 6.92–6.98 (m, 2H, arom. H), 7.02 (s, 1H-C(3)), 7.36 (dd, $J = 9.2$, 3.2, 1H-C(7)), 7.55 (d, $J = 3.2$, 1H-C(5)), 7.56–7.62 (m, 5H, arom. H), 7.71 (d, $J = 9.2$, 1H-C(8)), 8.07–8.10 (m, 2H, arom. H), 11.79 (s, NOH). ^{13}C NMR (100 MHz, DMSO-d_6): 55.10 (MeO), 59.30 (CH_2O), 105.85, 106.17, 113.72, 120.18, 123.41, 124.05, 126.27, 127.74, 129.08, 130.28, 131.17, 131.72, 150.58, 151.98, 155.16, 159.80, 162.30 (arom. C and C=N), 176.76 (C(4)). Anal. Calcd for $C_{24}H_{19}NO_5$: C 71.81, H 4.77, N 3.49. Found: C 71.75, H 4.78, N 3.49.

5.1.9. (*E*)-3-(2-Methoxyiminopropoxy)-2-phenyl-4*H*-1-benzopyran-4-one (13). To a solution of **1** (0.29 g, 1 mmol) in EtOH (20 ml) was added a solution of *O*-methylhydroxylamine hydrochloride (0.17 g, 2 mmol) in EtOH (2 ml). The mixture was heated at reflux for 24 h (TLC monitoring) and evaporated to give a residual solid. The white solid thus obtained was collected and purified by FC (silica gel; CH_2Cl_2) and recrystallized from ether to give **13** (0.22 g, 68 %). mp 107–108 °C. ^1H NMR (400 MHz, DMSO-d_6): 1.66 (s, Me), 3.71 (s, MeO), 4.53 (s, OCH_2), 7.52–7.54 (m, 1H, arom. H), 7.58–7.60 (m, 3H, arom. H), 7.74–7.76 (m, 1H, arom. H), 7.82–7.84 (m, 1H, arom. H), 7.99–8.01 (m, 2H, arom. H), 8.10–8.13 (m, 1H, arom. H). ^{13}C NMR (100 MHz, DMSO-d_6): 12.96 (Me), 61.88 (MeO), 73.43 (CH_2O), 119.19, 124.61, 125.68, 125.91, 129.23, 129.44, 130.98, 131.70, 134.91, 139.72, 153.95, 155.49, 156.72 (arom. C and C=N), 174.40 (C(4)). Anal. Calcd for $C_{19}H_{17}NO_4$: C 70.58, H 5.30, N 4.33. Found: C 70.56, H 5.31, N 4.28.

The same procedure was applied to convert **2** to **14**; **3** to **15**; **5a–c** to **21a–c**; **6a–c** to **22a–c**; and **7a–c** to **23a–c**, respectively.

5.1.10. (*E*)-6-(2-Methoxyiminopropoxy)-2-phenyl-4*H*-1-benzopyran-4-one (14). Yield: 77%. mp 113–114 °C. ^1H NMR (400 MHz, DMSO-d_6): 1.88 (s, Me), 3.82 (s, MeO), 4.68 (s, OCH_2), 7.02 (s, 1H-C(3)), 7.47 (dd, $J = 8.8$, 3.2, 1H-C(7)), 7.51 (d, $J = 3.2$, 1H-C(5)), 7.56–7.61 (m, 3H, arom. H), 7.77 (d, $J = 8.8$, 1H-C(8)), 8.08–8.11 (m, 2H, arom. H). ^{13}C NMR (100 MHz, DMSO-d_6): 12.80 (Me), 62.05 (MeO), 70.17 (CH_2O), 106.87, 107.09, 120.95, 124.41, 124.70, 127.00, 129.82, 131.86, 132.48, 151.34, 153.77, 156.01, 163.08 (arom. C and C=N), 177.51 (C(4)). Anal. Calcd for $C_{19}H_{17}NO_4$: C 70.58, H 5.30, N 4.33. Found: C 70.24, H 5.27, N 4.28.

5.1.11. (*E*)-7-(2-Methoxyiminopropoxy)-2-phenyl-4*H*-1-benzopyran-4-one (15). Yield: 84%. mp 100–101 °C. ^1H NMR (400 MHz, DMSO-d_6): 1.90 (s, Me), 3.83 (s, MeO), 4.73 (s, OCH_2), 6.95 (s, 1H-C(3)), 7.10 (dd, $J = 8.8$, 2.4, 1H-C(6)), 7.36 (d, $J = 2.4$, 1H-C(8)), 7.56–7.60 (m, 3H, arom. H), 7.95 (d, $J = 8.8$, 1H-C(5)), 8.06–8.08 (m, 2H, arom. H). ^{13}C NMR (100 MHz,

DMSO-d_6): 12.88 (Me), 62.09 (MeO), 70.28 (CH_2O), 102.72, 107.51, 115.66, 118.19, 126.88, 126.99, 129.79, 131.81, 132.39, 153.44, 157.97, 162.95, 163.21 (arom. C and C=N), 177.11 (C(4)). Anal. Calcd for $C_{19}H_{17}NO_4$: C 70.58, H 5.30, N 4.33; Found: C 70.42, H 5.32, N 4.30.

5.1.12. (*Z*)-3-(2-Methoxyimino-2-phenylethoxy)-2-phenyl-4*H*-1-benzopyran-4-one (21a). Yield: 77%. mp 109–110 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 3.80 (s, MeO), 5.20 (s, OCH_2), 7.34–7.37 (m, 3H, arom. H), 7.43–7.54 (m, 4H, arom. H), 7.73–7.93 (m, 6H, arom. H), 8.14–8.16 (m, 1H, arom. H). ^{13}C NMR (100 MHz, DMSO-d_6): 62.66 (CH_2O), 63.14 (MeO), 119.14, 124.08, 125.68, 125.91, 127.34, 128.91, 129.01, 129.24, 129.97, 130.73, 131.54, 134.30, 134.93, 140.03, 153.96, 155.44, 156.33 (arom. C and C=N), 174.65 (C(4)). Anal. Calcd for $C_{24}H_{19}NO_4$: C 74.79, H 4.97, N 3.63. Found: C 74.77, H 5.01, N 3.57.

5.1.13. (*Z*)-3-[2-(4-Fluorophenyl)-2-methoxyiminoethoxy]-2-phenyl-4*H*-1-benzopyran-4-one (21b). Yield: 86%. mp 135–136 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 3.79 (s, MeO), 5.19 (s, OCH_2), 7.12–7.17 (m, 2H, arom. H), 7.42–7.52 (m, 4H, arom. H), 7.74–7.91 (m, 6H, arom. H), 8.13–8.15 (m, 1H, arom. H). ^{13}C NMR (100 MHz, DMSO-d_6): 62.68 (CH_2O), 63.03 (MeO), 115.74, 115.96, 119.14, 124.05, 125.67, 125.91, 128.98, 129.24, 129.52, 129.60, 130.68, 130.74, 131.52, 134.94, 139.93, 153.13, 155.45, 156.40, 162.17, 164.62 (arom. C and C=N), 174.63 (C(4)). Anal. Calcd for $C_{24}H_{18}FNO_4$: C 71.46, H 4.50, N 3.47. Found: C 71.53, H 4.61, N 3.51.

5.1.14. (*Z*)-3-[2-Methoxyimino-2-(4-methoxyphenyl)ethoxy]-2-phenyl-4*H*-1-benzopyran-4-one (21c). Yield: 62%. mp 95–96 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 3.76 (s, MeO), 3.77 (s, NOMe), 5.17 (s, OCH_2), 6.88–6.90 (m, 2H, arom. H), 7.44–7.54 (m, 4H, arom. H), 7.69–7.95 (m, 6H, arom. H), 8.14–8.16 (m, 1H, arom. H). ^{13}C NMR (100 MHz, DMSO-d_6): 55.86 (MeO), 62.48 (CH_2O), 63.02 (NOMe), 114.37, 119.15, 124.09, 125.09, 125.90, 126.67, 128.78, 129.00, 129.25, 130.76, 131.52, 134.93, 140.06, 153.43, 155.45, 156.32, 160.82 (arom. C and C=N), 174.69 (C(4)). Anal. Calcd for $C_{25}H_{21}NO_5$: C 72.28, H 5.10, N 3.37. Found: C 72.14, H 5.07, N 3.30.

5.1.15. (*Z*)-6-(2-Methoxyimino-2-phenylethoxy)-2-phenyl-4*H*-1-benzopyran-4-one (22a). Yield: 67%. mp 135–136 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 4.05 (s, MeO), 5.37 (s, OCH_2), 7.01 (s, 1H-C(3)), 7.33 (dd, J = 9.2, 3.2, 1H-C(7)), 7.39–7.40 (m, 3H, arom. H), 7.53 (d, J = 3.2, 1H-C(5)), 7.57–7.67 (m, 5H, arom. H), 7.71 (d, J = 9.2, 1H-C(8)), 8.07–8.09 (m, 2H, arom. H). ^{13}C NMR (100 MHz, DMSO-d_6): 60.47 (CH_2O), 63.08 (MeO), 106.41, 106.90, 121.00, 124.28, 124.70, 127.00, 127.46, 129.08, 129.82, 130.20, 131.85, 132.47, 133.66, 151.38, 154.31, 155.62, 163.09 (arom. C and C=N), 177.46 (C(4)). Anal. Calcd for $C_{24}H_{19}NO_4$: C 74.79, H 4.97, N 3.63. Found: C 74.42, H 5.13, N 3.53.

5.1.16. (*Z*)-6-[2-(4-Fluorophenyl)-2-methoxyiminoethoxy]-2-phenyl-4*H*-1-benzopyran-4-one (22b). Yield: 68%. mp 159–160 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 4.05 (s, MeO), 5.36 (s, OCH_2), 7.00 (s, 1H-C(3)), 7.20–7.25 (m, 2H, arom. H), 7.32 (dd, J = 9.2, 3.2, 1H-C(7)), 7.51 (d, J = 3.2, 1H-C(5)), 7.56–7.60 (m, 3H, arom. H), 7.68–7.71 (m, 2H, arom. H), 7.71 (d, J = 9.2, 1H-C(8)), 8.06–8.08 (m, 2H, arom. H). ^{13}C NMR (100 MHz, DMSO-d_6): 60.46 (CH_2O), 63.12 (MeO), 106.42, 106.90, 115.96, 116.19, 120.99, 124.24, 124.70, 126.99, 129.72, 129.79, 130.08, 130.11, 131.84, 132.45, 151.38, 153.50, 155.50, 162.26, 163.06, 164.71 (arom. C and C=N), 177.42 (C(4)). Anal. Calcd for $C_{24}H_{18}FNO_4$: C 71.46, H 4.50, N 3.47. Found: C 71.60, H 4.68, N 3.52.

5.1.17. (*Z*)-6-[2-Methoxyimino-2-(4-methoxyphenyl)ethoxy]-2-phenyl-4*H*-1-benzopyran-4-one (22c). Yield: 82%. mp 135–136 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 3.75 (s, MeO), 4.02 (s, NOMe), 5.32 (s, OCH_2), 6.93–6.95 (m, 2H, arom. H), 7.00 (s, 1H-C(3)), 7.33 (dd, J = 9.2, 3.2, 1H-C(7)), 7.52 (d, J = 3.2, 1H-C(5)), 7.55–7.62 (m, 5H, arom. H), 7.69 (d, J = 9.2, 1H-C(8)), 8.06–8.08 (m, 2H, arom. H). ^{13}C NMR (100 MHz, DMSO-d_6): 55.86 (MeO), 60.33 (CH_2O), 62.89 (NOMe), 106.38, 106.89, 114.50, 120.95, 124.21, 124.71, 125.95, 126.98, 128.87, 129.79, 131.85, 132.43, 151.34, 153.72, 155.63, 160.93, 163.02 (arom. C and C=N), 177.43 (C(4)). Anal. Calcd for $C_{25}H_{21}NO_5$: C 72.28, H 5.10, N 3.37. Found: C 71.93, H 5.11, N 3.38.

5.1.18. (*Z*)-7-(2-Methoxyimino-2-phenylethoxy)-2-phenyl-4*H*-1-benzopyran-4-one (23a). Yield: 82%. mp 135–136 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 4.05 (s, MeO), 5.39 (s, OCH_2), 6.96 (s, 1H-C(3)), 7.01 (dd, J = 8.8, 2.4, 1H-C(6)), 7.37 (d, J = 2.4, 1H-C(8)), 7.40–7.42 (m, 3H, arom. H), 7.57–7.59 (m, 3H, arom. H), 7.66–7.69 (m, 2H, arom. H), 7.92 (d, J = 8.8, 1H-C(5)), 8.06–8.09 (m, 2H, arom. H). ^{13}C NMR (100 MHz, DMSO-d_6): 60.65 (CH_2O), 63.07 (MeO), 102.44, 107.51, 115.39, 118.27, 126.87, 127.07, 127.41, 129.17, 129.82, 130.31, 131.80, 132.40, 133.62, 153.81, 157.98, 162.91, 162.92 (arom. C and C=N), 177.07 (C(4)). Anal. Calcd for $C_{24}H_{19}NO_4$: C 74.79, H 4.97, N 3.63. Found: C 74.64, H 4.95, N 3.58.

5.1.19. (*Z*)-7-[2-(4-Fluorophenyl)-2-methoxyiminoethoxy]-2-phenyl-4*H*-1-benzopyran-4-one (23b). Yield: 86%. mp 160–161 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 4.04 (s, MeO), 5.40 (s, OCH_2), 6.97 (s, 1H-C(3)), 7.01 (dd, J = 8.8, 2.4, 1H-C(6)), 7.23–7.28 (m, 2H, arom. H), 7.37 (d, J = 2.4, 1H-C(8)), 7.58–7.61 (m, 3H, arom. H), 7.71–7.74 (m, 2H, arom. H), 7.92 (d, J = 8.8, 1H-C(5)), 8.07–8.10 (m, 2H, arom. H). ^{13}C NMR (100 MHz, DMSO-d_6): 60.69 (CH_2O), 63.11 (MeO), 102.51, 107.53, 115.38, 116.07, 116.29, 118.32, 126.88, 127.08, 129.72, 129.82, 130.06, 130.09, 131.81, 132.41, 153.06, 157.97, 162.33, 162.82, 162.93, 164.78 (arom. C and C=N), 177.07 (C(4)). Anal. Calcd for $C_{24}H_{18}FNO_4$: C 71.46, H 4.50, N 3.47. Found: C 71.07, H 4.69, N 3.46.

5.1.20. (*Z*)-7-[2-Methoxyimino-2-(4-methoxyphenyl)ethoxy]-2-phenyl-4*H*-1-benzopyran-4-one (23c). Yield: 85%. mp 157–158 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 3.77 (s, MeO), 4.02 (s, NOMe), 5.36 (s, OCH_2), 6.95–6.97 (m, 2H, arom. H), 6.98 (s, 1H-C(3)), 7.02 (dd, J = 8.8, 2.4, 1H-C(6)), 7.38 (d, J = 2.4, 1H-C(8)), 7.58–7.64 (m,

5H, arom. H), 7.92 (d, $J = 8.8$, 1H-C(5)), 8.07–8.10 (m, 2H, arom. H). ^{13}C NMR (100 MHz, DMSO-d_6): 55.90 (MeO), 60.52 (CH_2O), 62.88 (NOMe), 102.39, 107.52, 114.61, 115.43, 118.23, 125.90, 126.87, 127.06, 128.84, 129.82, 131.80, 132.42, 153.26, 158.00, 161.03, 162.92, 162.95 (arom. C and C=N), 177.08 (C(4)). Anal. Calcd for $C_{25}H_{21}NO_5$: C 72.28, H 5.10, N 3.37. Found: C 72.04, H 5.11, N 3.33.

5.2. Antiproliferative activity

5.2.1. Cell culture. Human cervical epithelioid carcinoma HeLa, hepatocellular carcinoma SKHep1, and Oral squamous cell carcinoma SAS were purchased from Bioresources Collection and Research Center, Taiwan. Cell line was maintained in the same standard medium and grown as a monolayer in DMEM (Gibco, USA) and supplemented with 10% fetal bovine serum (FBS) and antibiotics, i.e., 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin. Culture was maintained at 37 °C with 5% CO_2 in a humidified atmosphere.

5.2.2. Antiproliferative assay. Cancer cells were treated as indicated for 48 h in medium containing 10% FBS. (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, 2 mg/ml) (MTT, 20 ml) was added to the cultures and incubated during the final 1.5 h. The resultant tetrazolium salt was then dissolved by the addition of dimethyl sulfoxide. Color was measured spectrophotometrically in a microtiter plate reader at 570 nm and used as a relative measure of viable cell number. The number of viable cells following treatment was compared to solvent and untreated control cells and used to determine the percent of control growth as $(Ab_{treated}/Ab_{control}) \times 100$, where Ab represents the mean absorbance ($n = 3$). The concentration that killed 50% of cells (GI_{50}) was determined from the linear portion of the curve by calculating the concentration of agent that reduced absorbance in treated cells, compared to control cells, by 50%.

5.2.3. Flow cytometric analysis. HeLa cells treated with DMSO or **18c** at a concentration of 5 μM for 8 or 24 h were harvested, rinsed in PBS, resuspended and fixed in 80% ethanol, and stored at −20 °C in fixation buffer until ready for analysis. Then the pellets were suspended in 1 ml propidium iodide (PI) solution containing 20 μg/μl PI, 0.2 mg/ml RNase, and 0.1% (v/v) Trition X-100. Cell samples were incubated at room temperature in the dark for at least 30 min and analyzed by a FACScan flow cytometer (Becton–Dickinson, Mountain View, CA). Data recording was made using CELLQuest software (Becton–Dickinson, Mountain View, CA) and cell cycle data were analyzed using ModFitLT software (Veruty Software House, USA).

5.3. Antiplatelet evaluation

The following reagents were used: collagen (type 1, bovine Achilles tendon; from Sigma) was homogenized in 25 mM AcOH and stored (1 mg/ml) at −70°. Arachidonic acid (AA), EDTA (*N*,*N*,*N*′,*N*′-ethylenediamine tetraacetate), and bovine serum albumin (BSA) were purchased from Sigma and dissolved in $CHCl_3$. To test platelet aggregation, blood was collected from the rabbit marginal-ear vein, anticoagulated with EDTA (6 mM), and centrifuged for 10 min at 90*g* at rt Platelet suspensions were prepared from the plasma according to a washing procedure previously described.[24] Platelet numbers were determined with a Coulter ZM counter, and adjusted to 4.5×10^8 platelets/ml. The platelet pellets were suspended in Tyrode's solution of the following composition (in mM): NaCl (136.8), KCl (2.8), $NaHCO_3$ (11.9), $MgCl_2$ (2.1), NaH_2PO_4 (0.33), $CaCl_2$ (1.0), and glucose (11.2) containing BSA (0.35%). The platelet suspension was stirred at 1200 rpm, and the aggregation was measured at 37 °C by the turbidimetric method described by O'Brien,[25] using a Chrono Log Lumi aggregometer. To eliminate solvent effects, the final concentration of dimethylsulfoxide (DMSO) was fixed at 0.5%. The percentage of aggregation was calculated based on the absorbances of a platelet suspension and that of Tyrode's solution, which were taken as 0% and 100% aggregated, respectively.

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