Accepted Manuscript

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PII: S0223-5234(18)30939-5

DOI: https://doi.org/10.1016/j.ejmech.2018.10.062

Reference: EJMECH 10848

To appear in: *European Journal of Medicinal Chemistry*

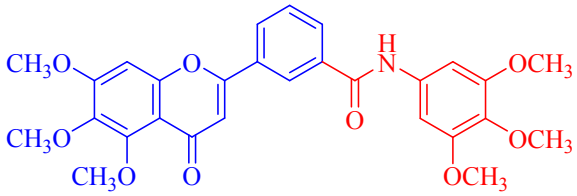
Received Date: 9 August 2018

Revised Date: 25 October 2018

Accepted Date: 28 October 2018

Please cite this article as: A.H.E. Hassan, E. Choi, Y.M. Yoon, K.W. Lee, S.Y. Yoo, M.C. Cho, J.S. Yang, H.I. Kim, J.Y. Hong, J.-S. Shin, K.-S. Chung, J.-H. Lee, K.-T. Lee, Y.S. Lee, Natural products hybrids: 3,5,4′-trimethoxystilbene-5,6,7-trimethoxyflavone chimeric analogs as potential cytotoxic agents against diverse human cancer cells, *European Journal of Medicinal Chemistry* (2018), doi: https://doi.org/10.1016/j.ejmech.2018.10.062.

Graphical Abstract:



4f

% Growth inhibition at 10 µM

RPMI8226 = 99.70

MDAMB435 = 93.64%

SKMEL5 = 91.66%

HS578T = 90.80%

MOLT4 = 89.58%

UO31 = 79.10%

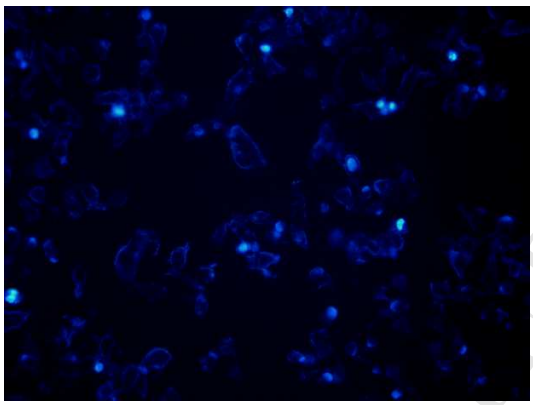
LOXIMVI = 75.56%

HOP92 = 75.26%

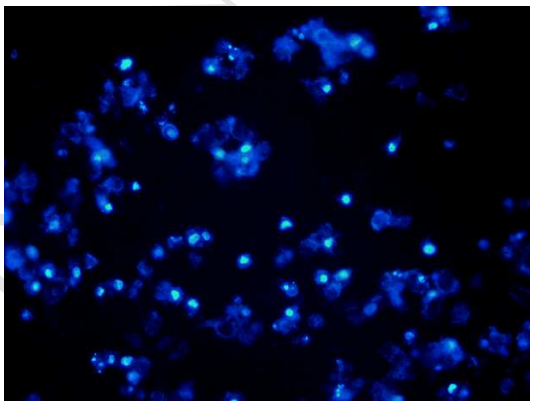
BT549 = 73.02%

K562 = 72.38%

Control



Compound 4f (10 µM)



Natural products hybrids: 3,5,4'-Trimethoxystilbene-5,6,7-trimethoxyflavone chimeric analogs as potential cytotoxic agents against diverse human cancer cells

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Abstract

Cancer still represents a major global health problem. All currently available anticancer agents have disadvantages like resistance or side effects. Therefore, introduction of novel anticancer agents is needed. Intrigued by the high success rate for natural products-based drug discovery, we designed and synthesized antiproliferative chemical entities as hybrids of two natural products; 3,5,4'-trimethoxystilbene and 5,6,7-trimethoxyflavone. To probe the spectrum of the synthesized compounds, *in vitro* evaluation was conducted against nine panels representing major cancer diseases. The results revealed the hybrid analogs **4f**, **4h**, **4k** and **4q** as promising broad-spectrum anticancer lead compounds eliciting high growth inhibition of several cell lines representing multiple cancers diseases. Evaluation of the promising lead compounds against normal human cell lines suggested a selective cytotoxic effect on cancer cells. Mechanistic investigation of the cytotoxic activity of compound **4f** in human cervical cancer HeLa cells showed that it triggers cell death through induction of apoptosis. As a whole, this study presents the natural products hybrid analogs **4f**, **4h**, **4k** and **4q** as potential lead compounds for further development of novel anticancer therapeutics.

***Keywords*:**

3,5,4'-Trimethoxystilbene; 5,6,7-Trimethoxyflavone; Chimeric molecules; Anticancer; Apoptosis.

1. Introduction

Ranked the second cause of death after cardiovascular diseases, cancer remains a major health problem. A recent analysis of the intrinsic and extrinsic factors inducing cancers revealed that up to 90% of cancers are caused by extrinsic factors like diet, lifestyle, pollution and infections [1]. Because many extrinsic predisposing factors for cancer incidence such as the modern life style, popularity of certain foods and pollution are anticipated to accrue in future, more people might be risked to cancer. The heterogeneity of cancers and evolving of resistance to the currently used anticancer drugs render them ineffective overtime and necessitate the development of novel anticancer agents to help in the fight against cancer.

Nature; the greatest invention machine, served as a plentiful source of drugs since old days [2]. A recent survey of anticancer drugs approved over 34 years from 1981 to 2014 revealed that 113 new chemical entities out of the 136 approved small molecules anticancer drugs were either natural products, derived from natural products or inspired by natural products [3]. Because of the high success rate for natural products-based drug discovery and development, we initiated our research to develop novel anticancer agents using the privileged scaffolds developed in the R&D lab of nature as starting points.

3,5,4'-Trimethoxystilbene (TMS; **1a**; Fig. 1) is a natural product that has been isolated from several plants [4-7]. It is a permethylated analog of resveratrol (RES; **1b**); another natural product that exists in a wide range of edible plants including vine grapes, legumes and berries [8]. Review of literature reports shows that RES (**1b**) has been the subject of several biological evaluations, as well as, clinical trials as a potential anticancer agent impacting several pathways that control the proliferation and survival of cells [9, 10]. However, the clinical benefit of RES

(**1b**) is not sufficiently confirmed [11]. In addition, it shows poor pharmacokinetics, as well as, chemical instability that hinder further development [12, 13]. On the other side, TMS (**1a**); the permethylated analog, is a more promising anticancer agent with an *in vitro* and *in vivo* proven better anticancer activity, as well as, enhanced pharmacokinetic properties relative to RES [14-16]. This stimulated researchers to investigate the cytotoxic activity of several TMS analogs bearing various methoxylation patterns [17-19]. According to published reports, TMS (**1a**) induces apoptosis and exhibits anticancer activity through multiple pathways including PI3K/Akt, Wnt/β-catenin, MAPK pathway and decrement of expression of matrix metalloproteinase-2 (MMP-2) [20-22]. Despite the premises that TMS and stilbenoid analogs are promising anticancer agents, the fact that such stilbenoid compounds are liable for *cis*/*trans* isomerization *in vivo* raises doubts about the anticipated *in vivo* activity and complicates further development of these compounds [23]. Although having much lower anticancer activity, isomerization is nullified in the reported amide analog (**2**) [24].

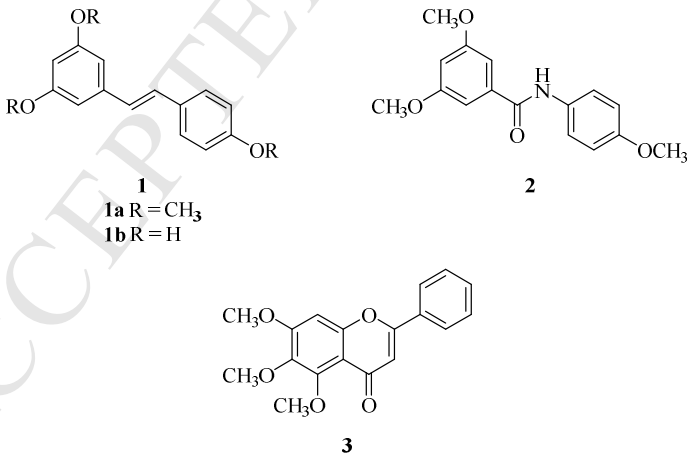


Figure 1. Reported molecules served as starting points for the conducted study

Natural flavones belong to an important class of medicinally privileged scaffold with reported diverse biological activities [25]. 5,6,7-Trimethoxyflavone (TMF; **3**; Fig. 1) is one of these

natural flavones that has been reported to be a constituent in the several plants [26-29]. At a concentration of 25 μM. TMF produces considerable cytotoxic effects in Hep G2, but to a lesser degree in Hep 3B and DU-145 cancer cells [30]. While the mechanism of the cytotoxic effects of TMF is understudied, it is known that TMF suppress nuclear factor-kappa B (NF-κB) which is a player in the development of cancer and inflammatory diseases [31-33]. In addition, *in vitro* evaluation of TMF showed that it inhibits topoisomerase II [34]. Introduction of various methoxylation patterns, hydroxy, nitro or amino moieties at B-ring of TMF provided compounds with almost similar potency to TMF [30]. This might indicate that the simple substitution is not sufficient to enhance the anticancer activity of TMF.

Combining the pharmacophoric features of two cytotoxic molecules into a single chemical entity has been established as a powerful strategy to develop more promising novel cytotoxic compounds that might elicit more than one mechanism of action [35, 36]. Accordingly, we thought of this molecular hybridization strategy to develop chimeric molecules **4** (Fig. 2) possessing the pharmacophoric features of both of the anticancer amide analog (**2**) of the natural products TMS and of the natural product TMF (**3**) in attempts to identify promising anticancer lead compounds. As shown in Fig. 2, the design involved replacement of one of the aromatic phenyl rings of TMS analog (**2)** with TMF (**3**). The natural product TMS has two phenyl rings, one has 4-methoxy substituent while the other has two methoxy substituents in 3,5-poistions. Because of this variation in both of the number of methoxy substituents and their positions on the phenyl rings, we wondered about the impact of such variations on the elicited biological activity in the designed compounds. Accordingly, various monomethoxylation, 3,4-methylenedioxy and 3,4,5-trimethoxy substitution patterns, as well as, the unsubstituted-phenyl moiety were planned to be explored as a pharmacophoric feature inherited from TMS. In the design, the attachment of

the amide moiety was established at 3-position of B-ring of TMF, meanwhile, the impact of the two possible amide configuration on the activity was planned to be assessed. In addition, the impact of switching the 5-methoxy substituent of the TMF moiety to 5-hydroxy was planned to be assessed. The latter was done as we encountered some reports claiming an enhancement of the cytotoxic activity of polymethoxylated flavones might happen upon selective demethylation of the 5-methoxy group [37, 38]. In following sections, we report our promising results.

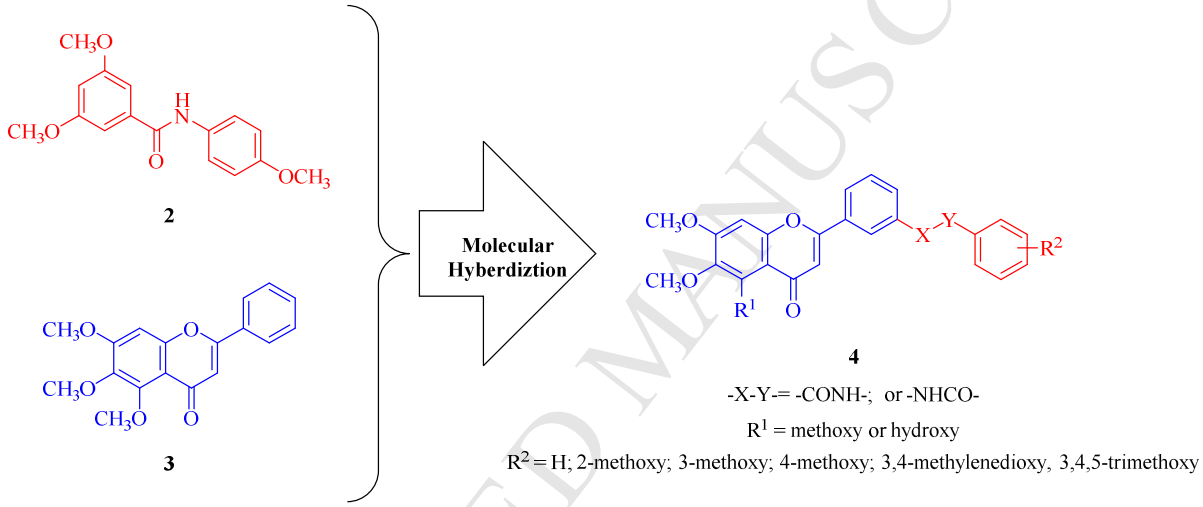


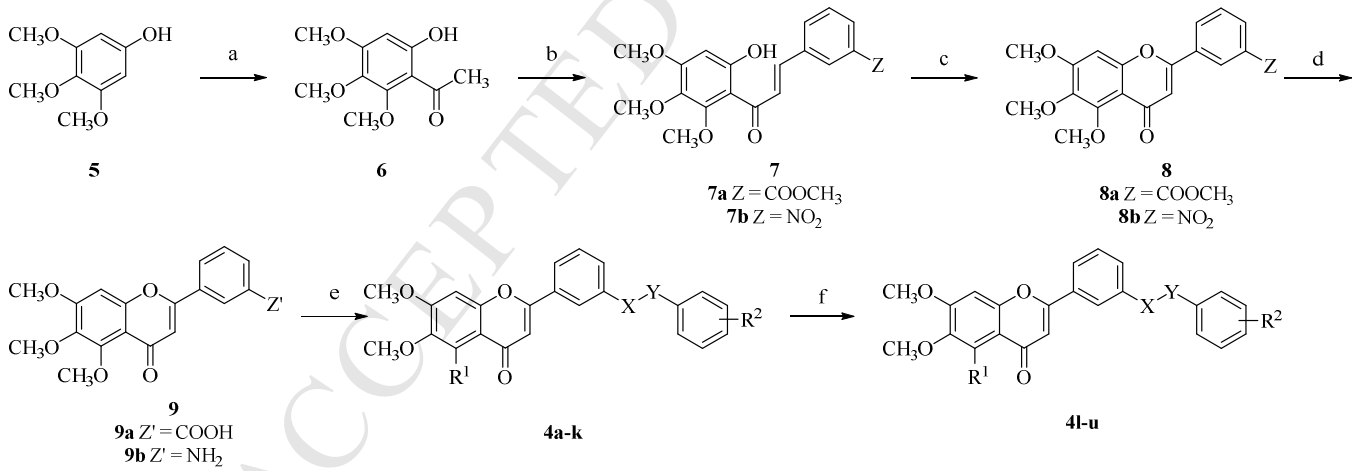
Figure 2. Design of TMS-TMF chimeric compounds

2. Results and discussion

2.1.Chemical Synthesis

As outlined in scheme 1, the targeted hybrid compounds (**4a**–**u**) were synthesized using modifications of the previously reported procedures [39-41]. First, 3,4,5-trimethoxyphenol (**5**) was acetylated using boron trifluoride diethyl etherate as a Lewis acid in the presence of a large excess of acetic anhydride, followed by Fries rearrangement to yield acetophenone derivative (**6**). Using sodium methoxide as a base and methanol as a solvent, compound **6** was condensed with the appropriate benzaldehyde to afford the corresponding chalcone derivative (**7**) that was

converted into a flavone derivative (**8**) in an intramolecular oxidative cyclization reaction using iodine/dimethyl sulfoxide mixture. The methyl ester group of compound **8a** was hydrolyzed into carboxylic group by alkaline hydrolysis using potassium hydroxide in methanol to provide carboxylic derivative **9a**, while the nitro group of compound **8b** was reduced into amino group using tin(II) chloride to provide the amino derivative **9b**. The carboxylic acid derivative (**9a**) was advanced to the desired designed hybrid compounds **4a**–**f** through amide coupling with the appropriate amine derivative after activation of the carboxylic group by *in situ* prepared reagent from trimethyl phosphite and iodine [40]. On the other side, the targeted hybrid compounds **4g**–**k** were accessed through reaction of the appropriate acid chloride with the amino derivative **9b** in the presence of potassium carbonate using acetone as a solvent. The desired 5-hydroxy derivatives **4l**–**u**, were prepared from the corresponding 5-methoxy derivatives **4a**–**k** via a regioselective 5-*O*-demethylation using boron trichloride in dichloromethane.



Scheme 1. Reagents and conditions: (a) Acetic anhydride, boron trifluoride diethyl etherate, reflux, overnight; (b) Methyl 3-formylbenzoate (For **7a**) or 3-nitrobenzaldehyde (For **7b**), NaOMe, MeOH, rt, overnight; (c) I_2, DMSO, 70 °C, overnight; (d) For **9a**: KOH, MeOH, reflux, overnight; For **9b**: $SnCl_2$, EtOH, reflux, 2.5 h; (e) For **4a**–**f**: $(MeO)_3P$, I_2, NEt_3, appropriate amine, CH_2Cl_2, 0 °C to rt, 3 h; For **4g**–**k**: K_2CO_3, acetone, appropriate acid chloride, rt;(f) BCl_3, CH_2Cl_2, 0 °C to rt, 3h.

2.2. Biological evaluations

2.2.1. Sulforhodamine B (SRB) assay for the antiproliferative activity

While MTT assay, which depend on the metabolic activity of living cells, is popular, the sulforhodamine B (SRB) assay, which measure the cellular proteins, has the advantages of better measurements proportionality with cell numbers and high sensitivity independent on cell line [42, 43]. In fact, national cancer institute (NCI) adopts SRB assays for screening the antiproliferative activity of candidate anticancer compounds. The synthesized hybrid compounds were assayed by NCI against human cancer cell lines representing nine major cancer diseases. The screening was performed at single dose of 10 µM to check the efficacy of tested compounds at this dose. The antiproliferative activity of imatinib (Gleevec®; a FDA-approved anticancer tyrosine kinase inhibitor drug marketed by Novartis pharma) against the used cell lines at the same single dose of 10 µM was retrieved from NCI database (NSC 759854) and used as a reference standard for comparison. The results were expressed as percent growth inhibition. The determined value reflects a cytostatic activity of a compound if it is ≤ 100%. When the evaluated compounds shows a cytocidal activity to cancer cells (lethality to cancer cells), the values exceeds 100% indicating that the remaining population of cancer cells decreased relative to the initially employed population. In the following sections, the interesting results are discussed.

2.2.1.1. *In vitro* evaluation of antiproliferative activity in hematologic cancers

Among childhood cancers, leukemia comes to the fore accounting for one third. In addition, it frequently afflicts elderly people too. Leukemia might be lymphoblastic or myeloid according the type of leukemic progenitor cells. In addition, it might be also divided into acute or chronic leukemia. In 2018, 60,300 new cases and 24,370 deaths because of leukemia are anticipated [44]. The designed hybrid compounds were evaluated against cell lines representing childhood and

adult leukemia of acute, chronic lymphoblastic and myeloid types. The results are shown in Table 1.

Table 1. % Growth inhibition of cell lines representing hematologic cancers by compounds **4a**–**u** and imatinib at 10 µM Conc.

Comp.	R^1	R^2	X-Y	CCRFCEM	HL60(TB)	K562	MOLT4	RPMI8226	SR
4a	MeO	H	CONH	35.13	12.70	29.56	43.20	59.43	35.94
4b	MeO	2-MeO	CONH	48.31	10.10	51.17	38.74	73.69	32.43
4c	MeO	3-MeO	CONH	59.46	31.29	59.69	51.49	83.05	58.14
4d	MeO	4-MeO	CONH	50.73	21.16	50.44	36.78	87.45	18.36
4e	MeO	3,4-CH_2O_2	CONH	36.58	30.28	25.79	35.20	79.10	52.37
4f	MeO	3,4,5-TriMeO	CONH	68.74	64.01	72.38	89.58	99.70	70.19
4g	MeO	H	NHCO	54.27	39.49	56.90	59.35	73.38	68.40
4h	MeO	2-MeO	NHCO	83.98	42.34	63.61	71.17	93.25	ND
4i	MeO	3-MeO	NHCO	56.13	14.76	62.03	53.31	83.18	ND
4j	MeO	4-MeO	NHCO	72.49	55.20	79.44	76.69	81.23	71.43
4k	MeO	3,4-CH_2O_2	NHCO	111.94	65.43	82.60	97.10	153.74	88.49
4l	OH	H	CONH	-0.19	7.30	2.93	0.26	16.50	4.29
4m	OH	2-MeO	CONH	39.80	9.41	22.15	27.94	45.41	21.51
4n	OH	3-MeO	CONH	34.70	17.49	16.93	24.21	53.57	47.66
4o	OH	4-MeO	CONH	16.00	-29.67	8.23	-2.73	7.48	19.21
4p	OH	3,4-CH_2O_2	CONH	2.77	5.71	5.51	-0.50	20.24	24.15
4q	OH	3,4,5-TriMeO	CONH	41.89	22.01	45.48	27.18	72.98	45.62
4r	OH	2-MeO	NHCO	5.53	0.60	15.48	6.77	21.63	16.50
4s	OH	3-MeO	NHCO	50.52	19.48	69.21	49.26	74.17	54.82
4t	OH	4-MeO	NHCO	11.95	15.39	11.93	19.11	25.21	20.83
4u	OH	3,4-CH_2O_2	NHCO	11.68	14.02	15.04	15.77	20.63	18.98
Imatinib				6.60	-6.80	ND	18.00	12.60	14.60

All data are reported as the average of duplicates.

As presented in Table 1, imatinib; the reference drug used for comparison, elicited no to low antiproliferative activity (≤ 18.00%) at the employed 10 µM dose. As illustrated in Fig. 3, most of the synthesized and evaluated hybrid compounds were much more effective relative to the reference imatinib. In general, compounds **4a**–**k** possessing TMF moiety showed higher activities relative to compounds **4l**–**u** possessing 6,7-dimethoxy-5-hydroxyflavone moiety. Among the used cell lines, RPMI8226; a plasma cell myeloma, was the most responsive, showing very high to average inhibition by all TMF derivatives. Among them, compounds **4k**

was lethal to RPMI8226 cell line (153.74% growth inhibition, Table 1). In addition, compounds **4f**, **4h**, **4d**, **4i**, **4c**, and **4j** in decreasing order respectively elicited an excellent inhibition within the range of 99.70.74~81.23%. Furthermore, compounds **4e**, **4b**, **4g** and **4a** in decreasing order respectively elicited an inhibition range of 79.10~59.43%. This indicates that the leukemic RPMI8226 cell line is highly susceptible to this class of compounds. On the other hand, only three compounds (**4s**, **4q** and **4n**) among the 6,7-dimethoxy-5-hydroxyflavone derivatives produced highly significant inhibition values of 74.17, 72.98 and 53.57%. Among the rest of evaluated leukemic cell lines, the growth of the chronic myelogenous leukemia K562 cell line and the childhood T acute lymphoblastic leukemia CCRFCEM cell line were also significantly inhibited by compounds possessing TMF moiety, but lesser than RPMI8226 cell line. As illustrated in Fig. 3, compound **4g**–**k** and **4r**–**u**, whose B-ring of the flavone moiety was attached to the linker's nitrogen atom, were generally more active against the employed leukemic cell lines relative to the corresponding compounds **4a**–**f** and **4m**–**p**; whose B-ring of the flavone moiety was attached to the linker's carbonyl group. In addition, the results revealed different profiles for the compounds bearing identical methoxylation pattern on the aromatic phenyl moiety retained from TMS on the activity, depending on the configuration of the amide linker. This indicates that the amide linker plays a crucial role than simply linking the two-pharmacophoric TMS and the flavone moieties. Thus, among TMF derivatives whose B-ring of the flavone moiety was linked to the carbonyl group of the linker, derivative **4f** bearing 3,4,5-trimethoxyphenyl moiety possessed a high efficacy showing an inhibition range of 99.70~64.01% for all evaluated leukemic cell lines. Meanwhile, derivatives **4e** and **4a** bearing 3,4-methylenedioxyphenyl and unsubstituted-phenyl moieties respectively were the least effective relative to other derivatives (Table 1). Among TMF derivatives **4b**–**d** bearing

monomethoxyphenyl moieties, compound **4c** having 3-methoxyphenyl moiety was the most active showing an inhibition range of 83.05~59.46% against five out the six evaluated leukemic cell lines. In contrast, when the configuration of the amide linker was reversed, derivative **4k** bearing 3,4-methylenedioxyphenyl moiety became the most active among TMF derivatives whose B-ring of the flavone moiety is linked to the linker's nitrogen atom (Fig. 3). Among TMF derivatives **4g**–**j** possessing the latter amide configuration and bearing monomethoxyphenyl moieties, compound **4j** bearing 4-methoxyphenyl moiety was the most active eliciting inhibition 81.23~55.20% against all of the evaluated six leukemic cell lines (Fig. 3).

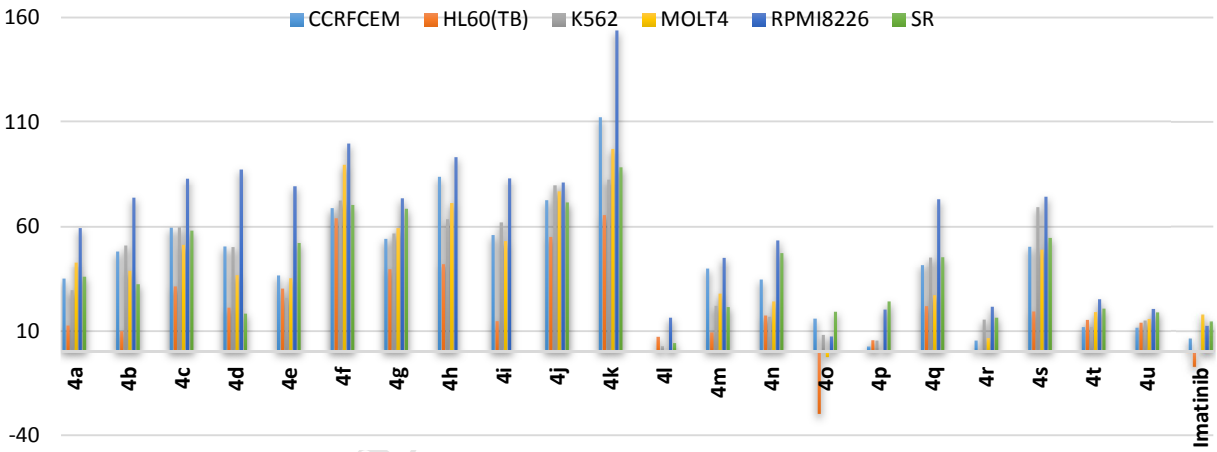


Figure 3. Percentage inhibition of growth inhibition of leukemic cells elicited by the synthesized TMS-TMF hybrid compounds and imatinib at a single dose of 10 µM.

2.2.1.2. *In vitro* evaluation of antiproliferative activity in non-small cell lung cancers

Lung adenocarcinoma, squamous cell lung carcinoma and large-cell lung carcinoma, collectively known as non-small cell lung cancers (NSCLC) are less sensitive to chemotherapeutic agents than the small lung cancers. Accordingly, NCI cancer cells panel incorporates several cell lines representing this difficult-to-treat class of lung cancers. The synthesized hybrid compounds were evaluated against these cells and the results are presented in Table 2.

Table 2. % Growth inhibition of cell lines representing non-small cell lung cancers by compounds **4a**–**u** and imatinib at 10 µM Conc.

Comp.	R^1	R^2	X-Y	A549	HOP62	HOP92	H226	H23	H322M	H460	H522
4a	MeO	H	CONH	16.66	13.71	29.18	12.73	7.79	17.66	11.22	21.89
4b	MeO	2-MeO	CONH	31.15	30.65	62.45	37.25	34.05	18.93	41.14	56.55
4c	MeO	3-MeO	CONH	37.21	40.50	58.14	36.69	36.87	19.91	39.89	53.37
4d	MeO	4-MeO	CONH	22.37	31.02	52.69	18.47	28.67	24.49	33.87	39.05
4e	MeO	3,4-CH_2O_2	CONH	25.51	26.63	21.94	21.19	10.27	22.56	26.91	26.87
4f	MeO	3,4,5-TriMeO	CONH	56.48	62.50	75.26	41.45	51.21	30.51	69.96	65.28
4g	MeO	H	NHCO	31.13	21.60	52.36	18.63	27.12	16.23	21.30	50.71
4h	MeO	2-MeO	NHCO	51.79	42.20	100.39	41.61	37.60	56.40	ND	55.74
4i	MeO	3-MeO	NHCO	44.06	30.25	51.80	28.02	33.94	27.12	ND	58.37
4j	MeO	4-MeO	NHCO	35.29	27.54	93.33	30.06	42.54	44.94	57.87	55.55
4k	MeO	3,4-CH_2O_2	NHCO	48.72	45.65	106.11	34.30	47.07	56.46	72.81	63.27
4l	OH	H	CONH	39.70	91.11	94.72	26.76	13.10	33.58	66.31	8.74
4m	OH	2-MeO	CONH	18.54	0.21	15.77	23.93	13.93	1.56	-3.83	26.65
4n	OH	3-MeO	CONH	26.63	37.53	33.59	41.48	25.42	12.86	31.39	59.84
4o	OH	4-MeO	CONH	18.74	27.78	76.39	8.07	8.74	0.54	7.30	20.56
4p	OH	3,4-CH_2O_2	CONH	25.13	39.65	26.99	21.48	27.32	19.03	45.73	23.95
4q	OH	3,4,5-TriMeO	CONH	59.46	88.33	43.85	92.31	65.14	25.62	46.22	58.77
4r	OH	2-MeO	NHCO	22.14	16.51	5.45	12.98	1.58	8.64	-2.21	40.98
4s	OH	3-MeO	NHCO	35.36	37.09	31.22	36.33	32.65	30.95	45.62	56.86
4t	OH	4-MeO	NHCO	15.12	30.20	42.34	41.43	15.66	8.16	17.35	34.41
4u	OH	3,4-CH_2O_2	NHCO	13.52	47.86	38.50	55.40	13.39	15.72	16.72	41.48
Imatinib				3.80	ND	43.70	10.60	17.10	ND	3.20	ND

All data are reported as the average of duplicates.

At the used 10 µM dose, imatinib; the standard drug used for comparison, elicited 43.70% growth inhibition against HOP92 cell line, while the determined growth inhibition percent of the rest of NSCLC cell lines was low (≤17.10%, Table 2). In comparison, several compounds among the synthesized and evaluated hybrid compounds were much more active relative to imatinib against the employed NSCLC cell lines as illustrated in Fig. 4. Depending on methoxylation pattern, configuration of amide linker and the switch between TMF and 6,7-dimethoxy-5-hydroxyflavone moieties, distinct profiles could be observed for the synthesized hybrid compounds. Comparing compounds **4a** and **4g** possessing unsubstituted-phenyl moiety as a pharmacophore inherited from TMS hybridized with TMF moiety, compound **4g** whose TMF

moiety was attached to linker's nitrogen atom was more active than compound **4a** whose TMF moiety was attached to the linker's carbonyl group (Fig. 4). In contrast to the decrement of activity that has been revealed in leukemic cell lines upon replacing TMF moiety of compound **4a** with 6,7-dimethoxy-5-hydroxyflavone moiety to afford compound **4l**, the activity profile was reversed in NSCLC cell lines as the compound **4l** showed much improved activity eliciting high inhibition of growth of lung adenocarcinoma HOP92 and HOP62 cell lines, as well as large cell lung carcinoma H460 cell line by 94.72, 91.11 and 66.31% respectively. This might indicate that the pathway(s) impacted by compound **4l** in NSCLC cells is almost inactive in leukemic cells. This activity pattern, which is the reverse to that observed in leukemic cell lines upon replacing TMF moiety with 6,7-dimethoxy-5-hydroxyflavone moiety, was also noted for compounds bearing the 3,4,5-trimethoxyphenyl moiety. As Fig. 4 shows, compound **4f** bearing the 3,4,5-trimethoxyphenyl was the most active among derivatives possessing TMF moiety attached to the carbonyl group of the amide linker. It elicited 75.26~51.21% inhibition against six cell lines out of the eight cell lines representing NSCLC. However, the corresponding compound **4q**, which possess 6,7-dimethoxy-5-hydroxyflavone moiety instead of TMF moiety, elicited a remarkable higher activity against some NSCLC cells lines. Thus, the growth of lung squamous carcinoma cell line H226 and lung adenocarcinoma cell line HOP62 were inhibited by 92.31 and 88.33% respectively. Again, this pattern of activity, which was absent in leukemic cells, might indicate the presence of a pathway(s) impacted by 6,7-dimethoxy-5-hydroxyflavone derivatives **4q** and **4l** in NSCLC, but absent in leukemic cells. Nevertheless, 6,7-dimethoxy-5-hydroxyflavone derivative **4m**–**p** and **4r**–**u** bearing monomethoxyphenyl or 3,4-methylenedioxyphenyl moieties were less active relative to the corresponding TMF derivatives **4b**–**e** and **4r**–**u**. As Fig. 4 illustrates, compounds **4r**–**u** whose TMF moiety was attached to the linker's nitrogen atom

possessed higher activity in general relative to compounds **4b**–**e** whose configuration of amide linker was the reverse. This emphasize amide linker as a crucial player in elicited activity rather than simply linking the two-combined pharamcophoric moieties. Among the evaluated NSCLC cell lines, the lung adenocarcinoma cell line HOP92 was the most sensitive to the synthesized hybrid compounds (Table 2). Its growth was inhibited by more than 100% by two compounds (**4k** and **4h**), within the range of 94.72~75.26% by four compounds (**4l**, **4j**, **4o** and **4f**) and within the range of 62.45~51.80% by five compounds (**4b**, **4c**, **4d**, **4g** and **4i**) which collectively accounts for eleven compounds out of the evaluated twenty-one compounds. Also, the lung adenocarcinoma cell line H522 was highly responsive to this class of compounds eliciting growth inhibition within the range of 65.28~50.71% by eleven compounds out of the evaluated twenty-one compounds (Table 2).

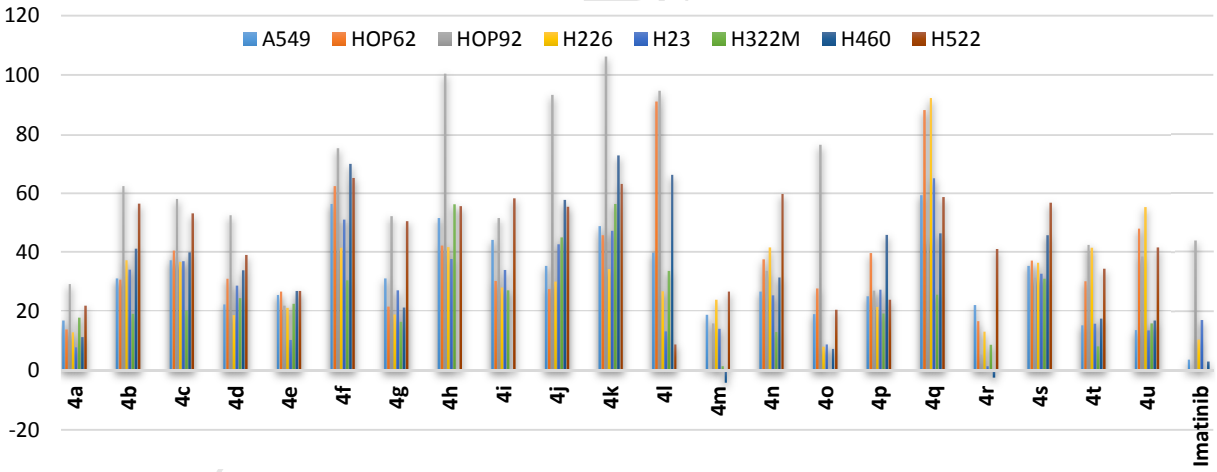


Figure 4. Percentage inhibition of growth inhibition of non-small cell lung cancer cells elicited by the synthesized TMS-TMF hybrid compounds and imatinib at a single dose of 10 µM.

2.2.1.3. *In vitro* evaluation of antiproliferative activity in colorectal cancers

Colorectal cancers' global incidence is anticipated to increase to more than 2.2 million cases and 1.1 million deaths by 2030 [45]. As of 2016, colorectal cancers are among the four most

common cancers in both men and women [46]. Furthermore, it was found that patients who had a colorectal cancer are prone to develop a second primary colorectal cancer unrelated to the first primary cancer [47]. Accordingly, the panel of cancer cell lines used for assay of the synthesized hybrid compounds by NCI included several cell lines representing colorectal adenocarcinoma. The outcome of the assay is summarized in Table 3.

Table 3. % Growth inhibition of cell lines representing colorectal cancers by compounds **4a–u** and imatinib at 10 µM Conc.

Comp.	R^1	R^2	X-Y	COLO205	HCC2998	HCT116	HCT15	HT29	KM12	SW620
4a	MeO	H	CONH	2.76	1.29	18.31	20.75	11.47	24.34	-0.72
4b	MeO	2-MeO	CONH	14.81	13.61	31.88	48.38	38.65	34.80	12.22
4c	MeO	3-MeO	CONH	31.68	12.23	39.28	45.33	49.17	46.30	10.40
4d	MeO	4-MeO	CONH	30.60	26.31	36.60	52.88	32.94	45.19	6.52
4e	MeO	3,4-CH_2O_2	CONH	24.39	2.03	27.32	28.24	17.21	32.67	-4.89
4f	MeO	3,4,5-TriMeO	CONH	55.70	25.88	51.40	59.30	68.95	54.24	33.02
4g	MeO	H	NHCO	15.64	12.19	32.28	34.82	22.94	35.58	8.49
4h	MeO	2-MeO	NHCO	ND	32.21	63.27	58.46	75.39	61.62	36.65
4i	MeO	3-MeO	NHCO	ND	19.52	46.44	46.65	53.63	53.93	22.76
4j	MeO	4-MeO	NHCO	26.91	29.67	52.19	54.76	53.26	53.71	11.98
4k	MeO	3,4-CH_2O_2	NHCO	31.88	46.90	64.53	67.89	70.59	48.72	36.02
4l	OH	H	CONH	7.73	10.27	53.74	7.23	45.85	42.23	5.67
4m	OH	2-MeO	CONH	-7.01	-2.45	14.48	12.96	10.83	0.81	-0.74
4n	OH	3-MeO	CONH	24.29	-3.54	30.73	25.70	15.64	35.48	18.36
4o	OH	4-MeO	CONH	2.39	-1.16	29.53	-7.36	12.32	-3.03	-1.58
4p	OH	3,4-CH_2O_2	CONH	5.22	-4.53	43.01	6.45	23.69	29.39	15.53
4q	OH	3,4,5-TriMeO	CONH	32.32	34.05	55.23	40.12	37.19	46.09	26.74
4r	OH	2-MeO	NHCO	-7.03	-9.63	11.68	3.51	2.63	5.13	10.54
4s	OH	3-MeO	NHCO	5.46	11.82	44.48	58.35	27.91	43.73	18.98
4t	OH	4-MeO	NHCO	-7.69	19.36	30.20	7.47	1.12	-0.05	9.52
4u	OH	3,4-CH_2O_2	NHCO	2.46	9.76	21.03	0.04	15.57	1.48	-1.42
Imatinib				-1.10	-22.00	18.60	11.50	47.10	-6.70	-10.00

All data are reported as the average of duplicates.

While imatinib inhibited the growth of HT29 colorectal cell line by 47.10%, it was ineffective at the 10 µm dose against four colorectal cell lines (COLO205, HCC2998, KM12 and SW620) and elicited only 18.60 and 11.50 against two cell lines (HCT116 and HCT15). As illustrated in Fig. 5, most of the evaluated compounds showed much better activity profiles relative to imatinib. In

general the percent growth inhibition induced by TMS analogs hybridized with TMF moiety (compounds **4a**–**k**) were higher than those triggered by corresponding TMS analogs hybridized with 6,7-dimethoxy-5-hydroxyflavone moiety (compounds **4l**–**u**). Considering the configuration of the amide linker, compounds **4g**–**k**, whose TMF moiety was attached to the linker's nitrogen atom, were more active relative to corresponding compounds whose TMF moiety is attached to the linker's carbonyl group. Among the latter, TMS-TMF hybrid compounds **4f** possessing 3,4,5-trimethoxyphenyl moiety was prominently active eliciting 68.95~51.40% growth inhibition against five out of seven cell lines representing colorectal cancers (Table 3). On the other side, among compounds **4g**–**k**, compounds **4h** possessing 2-methoxyphenyl moiety was prominently active compound eliciting 75.39~58.46% growth inhibition against four out of the six evaluated cell lines. In addition, compound **4j** bearing 4-methoxyphenyl moiety elicited 54.76~52.19% growth inhibition against four out of the seven tested cell lines. Furthermore, compound **4k** having 3,4-methylenedioxyphenyl moiety was highly active eliciting growth inhibition against three cell lines within the range of 70.59~64.53% while the corresponding compound **4e** was much less active. These, collectively, emphasize the influence of the amide linker configuration on the elicited activity. Replacement of TMF moiety of compound **4a** possessing unsubstituted-phenyl moiety as pharmacophore from TMS, by 6,7-dimethoxy-5-hydroxyflavone moiety in the corresponding compound **4l** resulted in some enhancement of activity. However, similar replacement of TMF in compounds **4b**–**k** possessing methoxylated-phenyl moieties, by 6,7-dimethoxy-5-hydroxyflavone moiety in the corresponding compound **4m**–**u** resulted in less effective compounds. This might indicate difference between molecular targets in colorectal cells influenced by compounds **4a** and **4l** bearing unsubstituted-phenyl moiety and those molecular target influenced by compounds **4b**–**k** and **4m**–**u** bearing methoxylated-phenyl moieties.

Regarding the susceptibility of the employed colorectal cell lines to this class of compounds, HCC2998 and SW620 cell lines were the least responsive cell lines showing inhibition percent lower than 50% for all of the evaluated hybrid compounds **4a**–**u**. Meanwhile, HCT116 and HCT15 cell lines showed 67.89~51.40% growth inhibition by six compounds; and HT29 showed 75.39~53.26% growth inhibition by five compounds. Accordingly, HCT116, HCT15 and HT29 cell lines were more responsive than other employed colorectal cell lines to the evaluated hybrid compounds.

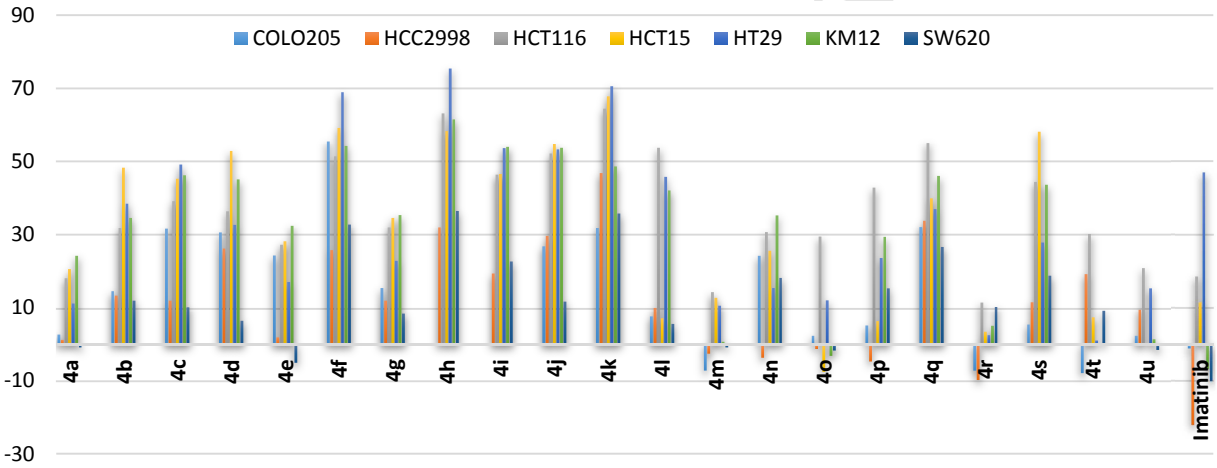


Figure 5. Percentage inhibition of growth inhibition of colon cancer cells elicited by the synthesized TMS-TMF hybrid compounds and imatinib at a single dose of 10 µM.

2.2.1.4. *In vitro* evaluation of antiproliferative activity in CNS cancers

Mortalities because of CNS cancers are the highest among other cancers mortalities in men and women before ages of 40 and 20 respectively [48]. Treatment of gliomas; the most common CNS cancers afflicting adults, is often hampered by molecular heterogeneity and limitations of drug's crossing of BBB [49]. Accordingly, the synthesized hybrid compounds were evaluated by NCI against six cell lines representing gliomas and the results are summarized in Table 4.

Table 4. % Growth inhibition of cell lines representing CNS cancers by compounds **4a**–**u** and imatinib at 10 µM Conc.

Comp.	R^1	R^2	X-Y	SF268	SF295	SF539	SNB19	SNB75	U251
4a	MeO	H	CONH	16.73	4.34	19.84	4.37	7.02	9.89
4b	MeO	2-MeO	CONH	29.33	38.21	30.51	17.52	30.05	11.03
4c	MeO	3-MeO	CONH	36.92	29.20	22.50	17.51	10.24	37.90
4d	MeO	4-MeO	CONH	27.86	33.62	27.66	8.54	19.73	13.86
4e	MeO	3,4-CH_2O_2	CONH	12.46	2.37	14.65	5.13	-1.27	9.36
4f	MeO	3,4,5-TriMeO	CONH	32.85	55.01	47.44	43.73	25.33	55.08
4g	MeO	H	NHCO	27.72	22.22	32.24	8.59	22.56	16.34
4h	MeO	2-MeO	NHCO	45.54	40.83	36.21	63.79	16.80	61.04
4i	MeO	3-MeO	NHCO	42.65	42.74	33.71	29.56	32.60	36.22
4j	MeO	4-MeO	NHCO	41.57	40.40	33.67	35.41	32.38	27.67
4k	MeO	3,4-CH_2O_2	NHCO	51.88	46.09	43.04	54.43	32.60	67.48
4l	OH	H	CONH	63.01	30.00	80.58	42.33	78.23	63.13
4m	OH	2-MeO	CONH	12.92	10.77	6.01	6.18	13.01	-0.16
4n	OH	3-MeO	CONH	22.91	17.64	20.74	16.39	1.94	23.94
4o	OH	4-MeO	CONH	45.38	-3.77	43.91	38.43	105.24	26.78
4p	OH	3,4-CH_2O_2	CONH	41.28	7.93	11.19	27.85	15.42	44.19
4q	OH	3,4,5-TriMeO	CONH	38.12	38.50	48.34	50.05	67.75	55.17
4r	OH	2-MeO	NHCO	8.58	6.16	9.52	25.37	16.45	33.08
4s	OH	3-MeO	NHCO	29.79	33.14	22.93	16.64	13.10	28.37
4t	OH	4-MeO	NHCO	13.90	20.63	15.31	34.11	32.53	28.93
4u	OH	3,4-CH_2O_2	NHCO	32.54	18.96	77.40	33.83	47.64	27.05
Imatinib				-3.80	15.10	24.50	-12.20	0.60	10.60

All data are reported as the average of duplicates.

At the used 10 µM dose, imatinib showed low growth inhibition against three cell lines (24.50, 15.10 and 10.60% for SF539, SF295 and U251 respectively) and was ineffective against another three cell lines (SNB19, SF268 and SNB75). As illustrated in Fig. 6, most of the evaluated compounds showed much better activity relative to imatinib. As illustrated in Fig. 6, compounds **4g**–**k**, whose TMF moiety was attached to the nitrogen atom of the linker, were more active relative to corresponding compounds **4a**–**e** whose TMF moiety was attached to the carbonyl group of the linker. As table 4 shows, compound **4g** possessing an unsubstituted-phenyl moiety elicited better activity, albeit low, relative to the corresponding compound **4a**. In addition, reversing the configuration of the amide linker of compounds **4b**/**4c** possessing 2-/3-methoxyphenyl moiety provided the corresponding compounds **4h** and **4i** eliciting more

enhanced activity. Thus, compound **4h** elicited 63.79 and 61.04% growth inhibition against SNB19 and U251 cell lines respectively. When the TMF moiety of compound **4a** was replaced by 6,7-dimethoxy-5-hydroxyflavone moiety, an abrupt increase of activity against multiple glioma cell lines was elicited by the corresponding compound **4l** (Fig. 6). Thus, compound **4l** inhibited SF539, SNB75, U251 and SF268 cell lines by 80.58, 78.23, 63.13 and 63.01% respectively. In contrast, replacement of the TMF moiety of compounds **4b**, **4c**, **4h**, **4i** or **4j** by 6,7-dimethoxy-5-hydroxyflavone moiety to afford the corresponding compound **4m**, **4n**, **4r**, **4s** or **4t** resulted in reduction of the activity. However, such replacement in compounds **4d** or **4e** having 4-methoxyphenyl moiety or 3,4-methylenedioxyphenyl to afford compounds **4o** or **4p** resulted in enhancement of activity against several cell lines representing gliomas. Noticeably, compound **4o** exerted a cytocidal effect on SNB75 glioma cell line inhibiting its growth by 105.24%. Despite the activity of compound **4f**, which have TMF and 3,4,5-trimethoxyphenyl moieties was prominent in leukemia, NSCLC and colorectal cancers, it was less prominent against the assessed CNS cancers. Thus, compound **4f** showed inhibition by more than 50% against only two cell lines out the six cell lines displayed in Table 4. Replacement of TMF moiety in compound **4f** by 6,7-dimethoxy-5-hydroxyflavone moiety afforded the corresponding compound **4q** showing approximately the same level of activity against most of the glioma cell lines evaluated. Among the evaluated glioma cell lines, SNB75 and SF539 cell lines showed the highest growth inhibition by two compounds for each within the ranges of 105.24~78.23% and 80.58~77.40% respectively. Meanwhile, U251 was responsive to the highest number of tested compounds eliciting growth inhibition by five evaluated compounds within the range of 67.48~55.08%.

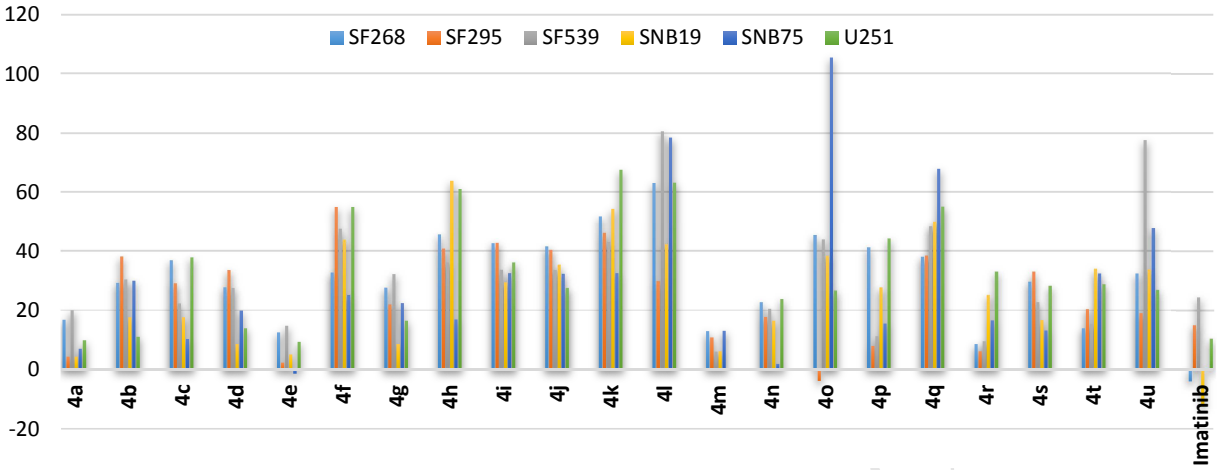


Figure 6. Percentage inhibition of growth inhibition of CNS cancer cells elicited by the synthesized TMS-TMF hybrid compounds and imatinib at a single dose of 10 µM.

2.2.1.5. *In vitro* evaluation of antiproliferative activity in melanoma

Among skin cancers, melanoma is the most dangerous, In 2018, the number of patients anticipated to be afflicted with melanoma in US only is 87,290 new cases [48]. The fact that in melanomas there is intratumor and intertumor heterogenicity undermine the therapeutic benefits of currently used anticancer agents [50, 51]. Accordingly, the synthesized hybrid compounds were evaluated by NCI against eight cell lines representing melanomas. The results are summarized in Table 5.

Table 5. % Growth inhibition of cell lines representing melanoma by compounds **4a**–**u** and imatinib at 10 µM Conc.

Comp	R^1	R^2	X-Y	LOXIMVI	MALME3M	MDAMB435	SKMEL2	SKMEL28	SKMEL5	UACC257	UACC62
4a	MeO	H	CONH	33.03	12.51	8.87	15.39	8.31	35.29	5.48	29.84
4b	MeO	2-MeO	CONH	52.09	22.85	22.39	14.39	24.55	58.35	-6.14	46.55
4c	MeO	3-MeO	CONH	50.15	15.15	18.83	24.30	26.58	36.13	7.62	37.89
4d	MeO	4-MeO	CONH	75.43	21.42	23.77	15.31	28.68	59.06	-2.76	35.23
4e	MeO	3,4-CH_2O_2	CONH	81.49	1.22	4.94	-2.38	20.39	47.23	5.96	19.13
4f	MeO	3,4,5-TriMeO	CONH	75.56	43.58	93.64	58.33	36.45	91.66	47.21	49.52
4g	MeO	H	NHCO	38.30	13.16	19.27	23.18	26.83	36.79	0.33	33.20
4h	MeO	2-MeO	NHCO	64.61	34.50	56.45	68.85	43.96	74.96	38.53	56.86
4i	MeO	3-MeO	NHCO	42.57	12.78	23.41	38.11	27.04	39.36	3.66	41.10
4j	MeO	4-MeO	NHCO	58.14	46.62	30.44	38.71	31.00	49.84	-4.69	44.57
4k	MeO	3,4-CH_2O_2	NHCO	60.51	23.57	46.70	37.64	44.87	83.60	22.71	50.01
4l	OH	H	CONH	11.89	35.47	41.72	35.04	12.57	28.29	15.72	61.09

4m	OH	2-MeO	CONH	16.66	5.55	2.77	14.05	5.62	3.77	1.19	26.52
4n	OH	3-MeO	CONH	36.76	12.33	22.61	2.64	14.23	26.82	8.74	44.42
4o	OH	4-MeO	CONH	20.25	19.38	-5.05	16.62	-4.12	8.61	20.81	18.93
4p	OH	3,4-CH_2O_2	CONH	11.79	11.90	20.87	10.11	10.23	16.04	21.82	18.57
4q	OH	3,4,5-TriMeO	CONH	67.01	49.05	34.63	30.39	22.57	60.83	33.92	50.65
4r	OH	2-MeO	NHCO	4.40	-12.70	1.26	0.56	-1.90	13.42	31.90	30.17
4s	OH	3-MeO	NHCO	57.56	29.49	33.62	21.56	22.50	48.93	25.86	39.09
4t	OH	4-MeO	NHCO	6.47	0.28	5.59	16.65	9.40	16.24	13.56	33.74
4u	OH	3,4-CH_2O_2	NHCO	5.74	7.67	5.39	6.66	15.22	25.85	17.18	41.40
Imatinib				11.60	-13.70	7.40	ND	-9.40	22.30	2.80	7.70

All data are reported as the average of duplicates.

At the used 10 µM dose, imatinib elicited 22.30, 11.60, 7.70 and 7.40% growth inhibition against SKMEL5, LOXIMVI, UACC62 and MDAMB435 cell lines respectively, while it was almost ineffective against MALME3M, SKMEL28 and UACC257 cell lines (Table 5). Relative to imatinib, several compounds among the synthesized and evaluated hybrid compounds were much more active against the employed melanoma cell lines as illustrated in Fig. 7. The results indicated that compound **4f**, which is a TMS-TMF hybrid compound having 3,4,5-trimethoxyphenyl moiety and an amide linker configuration in which the TMF moiety was attached to linker's carbonyl group, possessed an excellent activity against MDAMB435 and SKMEL5 cell lines evident by measured 93.64 and 91.66% growth inhibition against these two cell lines respectively. In addition, it elicited good inhibitory activity against LOXIMVI and SKMEL2 cell lines showing 75.56 and 58.33% growth inhibition respectively. The growth of the other three melanoma cell lines was also inhibited by compound **4f**, albeit with a lower inhibition percent. Thus, compound **4f** elicited the highest antiproliferative activity among all evaluated compounds against melanoma cell lines. When the same amide linker configuration was retained but the TMF moiety of compound **4f** was replaced with 6,7-dimethoxy-5-hydroxyflavone moiety to afford the corresponding compound **4q**, the elicited antiproliferative activity was reduced (Fig. 7). Also, replacing the 3,4,5-trimethoxyphenyl moiety of compound **4f** with unsubstituted-phenyl,

other methoxylated-phenyl or 3,4-methylenedioxyphenyl moiety while retaining the same amide linker configuration, resulted in the less active compounds **4a**–**e**. Replacement of the TMF moiety of compounds **4b**–**e** with 6,7-dimethoxy-5-hydroxyflavone moiety to afford the corresponding compounds **4m**–**p** resulted in further reduction of the elicited antiproliferative activity against the employed melanoma cell lines. In contrast, replacement of the TMF moiety of compound **4a** possessing unsubstituted-phenyl moiety with 6,7-dimethoxy-5-hydroxyflavone moiety to provide compound **4l** resulted in some enhancement of the antiproliferative activity (Fig. 7). This different behavior might indicate that the pathways contributing to the triggered growth inhibition are different in the case of compounds having unsubstituted-phenyl moiety from the cases of compounds having methoxylated-phenyl moieties. When the amide linker configuration of compounds **4a**–**e** was reversed to provide the corresponding compounds **4g**–**k**, the activity was enhanced (Fig. 7). Among the latter compounds, the 2-methoxyphenyl and the 3,4-methylenedioxyphenyl derivatives **4h** and **4k** elicited prominent antiproliferative activity (Fig. 7). Replacing the TMF moiety of compounds **4h**–**k** with 6,7-dimethoxy-5-hydroxyflavone moiety resulted in compounds **4r**–**u** with lower activity which is a similar behavior to that noted above for other tested compounds. The overall results showed that LOXIMVI and SKMEL5 were the most susceptible cell lines among the employed melanoma cell lines to the tested compounds while MALME3M, SKMEL28 and UACC257 were the least affected cell lines by these compounds

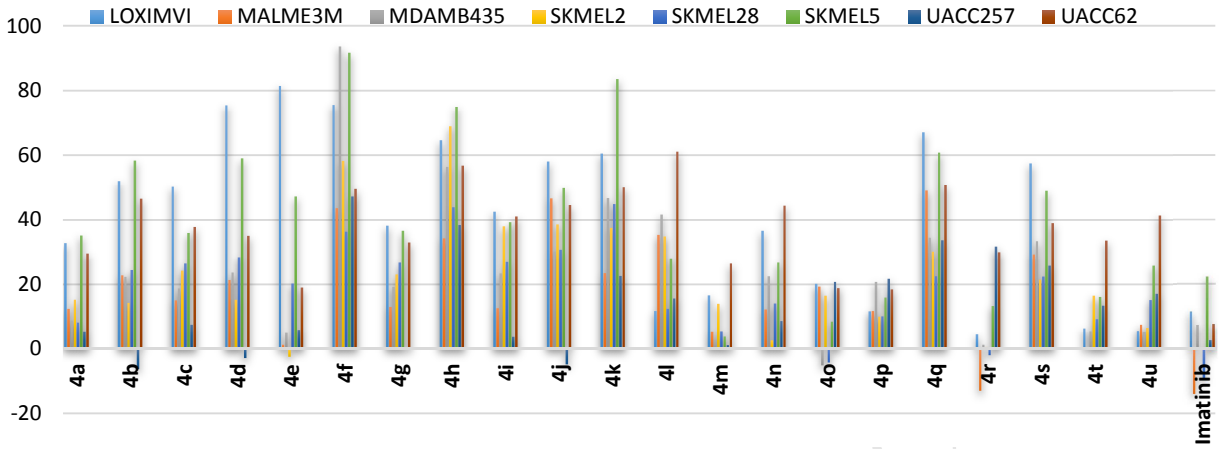


Figure 7. Percentage inhibition of growth inhibition of melanoma cells elicited by the synthesized TMS-TMF hybrid compounds and imatinib at a single dose of 10 µM.

2.2.1.6. *In vitro* evaluation of antiproliferative activity in ovarian cancers

Because the symptoms of ovarian cancers are more noticeable when the disease progress, most women diagnosed with ovarian cancers are in advanced stages which are difficult to cure [52]. Accordingly, development of novel therapeutics for ovarian cancers is extremely needed. The synthesized hybrid compounds were evaluated by NCI against a panel of cell lines representing ovarian cancers. The results are summarized in Table 6.

Table 6. % Growth inhibition of cell lines representing ovarian cancers by compounds **4a**–**u** and imatinib at 10 µM Conc.

Comp.	R^1	R^2	X-Y	IGROV1	OVCAR3	OVCAR4	OVCAR5	OVCAR8	ADRRES
4a	MeO	H	CONH	19.39	12.34	1.91	16.21	34.85	18.25
4b	MeO	2-MeO	CONH	27.52	47.51	19.41	18.65	42.67	46.70
4c	MeO	3-MeO	CONH	46.73	33.39	37.43	9.93	52.96	35.17
4d	MeO	4-MeO	CONH	38.66	54.26	15.33	16.77	46.12	47.21
4e	MeO	3,4-CH_2O_2	CONH	39.82	12.66	3.72	16.44	45.94	35.10
4f	MeO	3,4,5-TriMeO	CONH	48.21	59.16	25.15	13.97	69.12	48.93
4g	MeO	H	NHCO	21.64	15.81	18.28	-0.09	39.05	25.46
4h	MeO	2-MeO	NHCO	57.11	48.65	40.95	18.80	61.29	41.75
4i	MeO	3-MeO	NHCO	42.56	24.65	37.74	10.44	47.74	34.53
4j	MeO	4-MeO	NHCO	46.09	33.19	38.04	4.43	42.12	39.83
4k	MeO	3,4-CH_2O_2	NHCO	52.97	46.95	41.87	6.14	53.70	42.41
4l	OH	H	CONH	14.61	29.87	120.24	5.59	84.60	20.86

4m	OH	2-MeO	CONH	-5.97	-0.89	3.74	8.10	35.16	19.83
4n	OH	3-MeO	CONH	12.00	10.55	48.03	8.50	62.49	25.82
4o	OH	4-MeO	CONH	4.86	49.39	2.54	2.51	19.37	14.68
4p	OH	3,4-CH_2O_2	CONH	18.38	10.51	99.93	11.13	69.42	29.41
4q	OH	3,4,5-TriMeO	CONH	29.60	76.24	41.43	22.62	76.91	50.62
4r	OH	2-MeO	NHCO	3.04	-14.59	-5.24	5.27	17.48	5.26
4s	OH	3-MeO	NHCO	25.67	34.15	33.44	9.51	50.66	43.69
4t	OH	4-MeO	NHCO	2.38	-0.49	4.66	0.04	35.51	13.29
4u	OH	3,4-CH_2O_2	NHCO	5.56	-4.70	25.76	13.65	37.76	14.79
Imatinib				-18.60	-10.30	-0.10	-22.80	8.40	-0.50

All data are reported as the average of duplicates.

At the used 10 µM dose, imatinib was almost ineffective against the employed ovarian cancer cell lines. As shown in table 6, it did not inhibit the growth of all employed cell lines except for very weak inhibition of OVCAR8 by only 8.40%. In contrast, almost all of the synthesized and evaluated compounds possessed much better activities as illustrated in Fig. 8. The antiproliferative activity of both compounds **4a** and **4g**, which represent the two possible configurations of amide linked TMS-TMF hybrids possessing unsubstituted-phenyl moiety, was relatively weak against the employed ovarian cancer cell lines. However, replacement of TMF moiety with 6,7-dimethoxy-5-hydroxyflavone moiety while retaining the unsubstituted-phenyl moiety afforded compound **4l** showing a strikingly enhanced activity against two cell lines, as illustrated in Fig. 8. Thus, compound **4l** was lethal to OVCAR4 cell line (120.24% growth inhibition) and highly active against OVCAR8 cell line (84.60% growth inhibition). Comparing the activity of TMF derivatives **4b**–**e** and **4h**–**k** having reversed amide linker configuration-relationship reveals some enhancement of activity, albeit not high, for compounds whose TMF moiety was attached to the linker's nitrogen atom. In general, compounds **4f** and **4q** sharing the presence of 3,4,5-trimethoxyphenyl moiety were the most active among compounds having methoxylated-phenyl moieties. The activity of compound **4q** which possesses 6,7-dimethoxy-5-hydroxyflavone moiety was higher than compounds **4f** possessing TMF moiety. In

contrast to the noticed enhancement of activity upon replacing TMF moiety with 6,7-dimethoxy-5-hydroxyflavone moiety in derivatives **4l** and **4q**, compounds **4m**, **4n 4o**, **4r**, **4t** and **4u** having the 6,7-dimethoxy-5-hydroxyflavone moiety were in general less active than the corresponding compounds having the TMF moiety. Among the employed ovarian cancer cell lines, OVCAR8 was the most responsive to the evaluated compounds eliciting more than 50% inhibition by nine compounds out of the evaluated twenty-one compounds. Meanwhile, OVCAR5 was the least inhibited ovarian cancer cell line showing inhibition not more than 50% by all of the evaluated compounds.

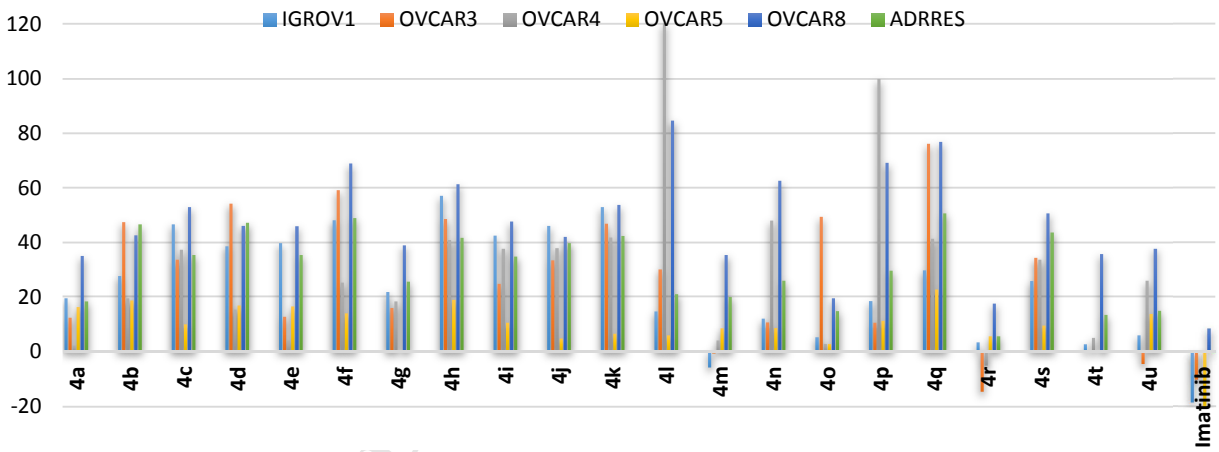


Figure 8. Percentage inhibition of growth inhibition of ovarian cancer cells elicited by the synthesized TMS-TMF hybrid compounds and imatinib at a single dose of 10 µM.

2.2.1.7. *In vitro* evaluation of antiproliferative activity in renal cancers

The global number of new renal cancer cases surpassed 350,000 in 2013 resulting in more than 140,000 deaths [53]. The rate of renal cancers incidence are increasing with the highest rates in the developed western countries [54]. The hybrid compounds were assessed by NCI against a panel of renal cancer cells and the results are presented in Table 7.

Table 7. % Growth inhibition of cell lines representing renal cancers by compounds **4a**–**u** and imatinib at 10 µM Conc.

Comp	R^1	R^2	X-Y	786-0	ACHN	CAKI1	RXF393	SN12C	TK10	UO31
4a	MeO	H	CONH	7.46	8.22	4.59	24.94	27.34	8.98	35.62
4b	MeO	2-MeO	CONH	39.19	43.80	43.00	29.26	34.60	10.41	69.58
4c	MeO	3-MeO	CONH	31.10	37.49	25.27	52.11	37.67	21.20	68.44
4d	MeO	4-MeO	CONH	29.33	33.37	63.66	59.88	34.99	15.72	56.15
4e	MeO	3,4-CH_2O_2	CONH	7.44	10.18	27.89	29.04	30.76	16.13	40.99
4f	MeO	3,4,5-TriMeO	CONH	68.25	43.33	59.37	46.78	54.08	30.59	79.10
4g	MeO	H	NHCO	21.85	38.36	12.77	25.64	23.81	8.72	48.96
4h	MeO	2-MeO	NHCO	54.66	57.02	34.88	40.97	58.95	46.56	84.75
4i	MeO	3-MeO	NHCO	47.22	42.08	18.06	28.94	38.69	17.21	66.69
4j	MeO	4-MeO	NHCO	45.04	53.03	42.56	46.43	50.03	23.32	73.51
4k	MeO	3,4-CH_2O_2	NHCO	80.49	52.24	22.93	53.51	57.91	38.37	75.77
4l	OH	H	CONH	68.53	41.96	51.13	43.46	25.16	39.23	40.34
4m	OH	2-MeO	CONH	9.66	9.27	6.75	21.77	5.99	14.08	30.03
4n	OH	3-MeO	CONH	14.23	33.80	32.38	25.82	18.96	6.27	41.69
4o	OH	4-MeO	CONH	56.97	16.39	6.41	8.01	15.71	-5.09	6.60
4p	OH	3,4-CH_2O_2	CONH	35.66	24.75	23.07	18.53	31.30	1.40	17.88
4q	OH	3,4,5-TriMeO	CONH	33.68	83.67	37.47	25.01	37.00	25.17	65.67
4r	OH	2-MeO	NHCO	11.35	5.14	9.48	-12.13	5.29	-0.86	15.19
4s	OH	3-MeO	NHCO	26.66	35.47	23.76	1.37	34.94	16.71	52.86
4t	OH	4-MeO	NHCO	24.53	23.74	20.04	4.27	25.67	18.42	4.63
4u	OH	3,4-CH_2O_2	NHCO	29.90	30.49	34.41	32.62	9.48	15.32	15.84
Imatinib				5.00	-1.00	-0.20	5.70	6.00	-10.00	7.60

All data are reported as the average of duplicates.

While the standard imatinib at the employed 10 µM dose elicited low activity (≤ 7.60%, Table 7), most of the evaluated compounds showed much better activity against the employed seven cell lines representing renal cancers (Fig. 9). In general, TMF derivatives **4g**–**k** in which the amide linker was configured so that the nitrogen atom of the linker was attached to the TMF moiety were more active than the corresponding compounds **4a**–**e** with the opposite linker configuration. As Table 7 shows, derivative **4h** and **4k** having 2-methoxyphenyl and 3,4-methylenedioxyphenyl moieties inhibited four cell lines by 84.75~54.66% and five cell lines by 75.77~52.24% respectively. In comparison, the corresponding derivatives **4b** and **4e** having linker configuration, in which the carbonyl group was attached to the TMF moiety were less effective as only one cell

line was inhibited by more than 50% by compound **4b** while none showed more than 50% inhibition by compound **4e**. Replacement of TMF moiety of the relatively weak compound **4a** which has unsubstituted-phenyl moiety, with 6,7-dimethoxy-5-hydroxyflavone moiety resulted in compound **4l** with more enhanced activity. Thus, while compound **4a** showed inhibition of the growth of 786-0 and CAKI1 cell lines by 7.46 and 4.59% respectively, compound **4l** showed inhibition of these two cell lines by 64.53 and 51.13 respectively. As noticed before in the assessments of antiproliferative activity, this enhancement of activity by replacing 6,7-dimethoxy-5-hydroxyflavone for TMF moiety in compounds with unsubstituted-phenyl moiety was reversed in compounds with monomethoxyphenyl moieties. Thus, compounds **4m**–**o** elicited much weaker activity relative to the corresponding compounds **4b**–**d**. In addition, replacing the TMF moiety of the highly active 3,4,5-trimethoxyphenyl derivative **4f** with 6,7-dimethoxy-5-hydroxyflavone moiety afforded the corresponding compound **4q** which was less active except against ACHN cell line (Table 7). This difference in the elicited activity upon replacing TMF with 6,7-dimethoxy-5-hydroxyflavone moiety might arise from presence of different active pathways in the different cells. Among active compounds possessing TMF moiety compounds **4f**, **4h**, **4j** and **4k** possessed prominent activity against several cell lines (Fig. 9). Also, the results showed that UO31 cell line as the most responsive among evaluated cell lines to the set of evaluated compounds showing growth inhibition within the range of 84.75~52.86% by ten compounds out of the evaluated twenty-one compounds. On the other hand, TK10 cells were the least affected cell line showing a maximum inhibition of 46.56% by the best inhibiting compound to this cell line.

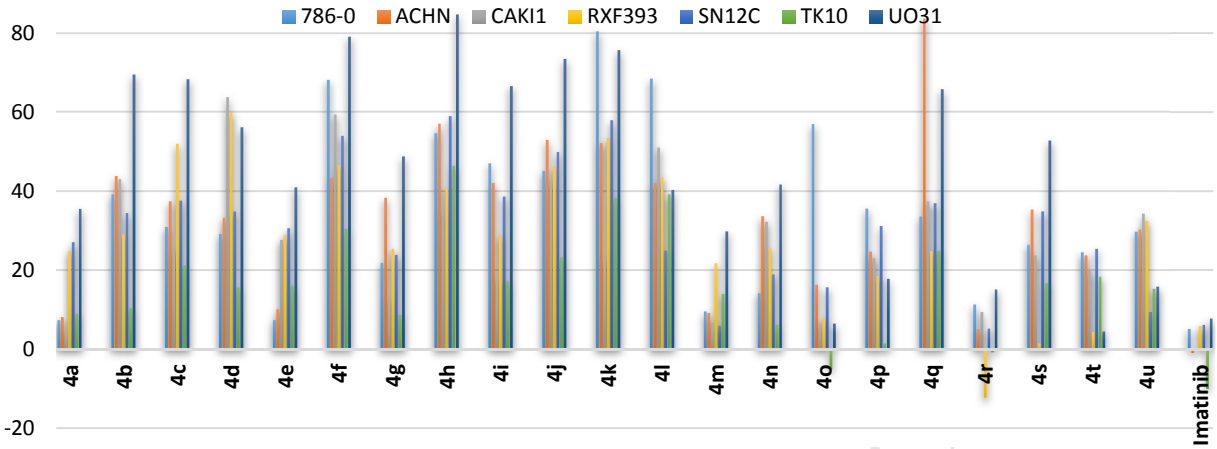


Figure 9. Percentage inhibition of growth inhibition of renal cancer cells elicited by the synthesized TMS-TMF hybrid compounds and imatinib at a single dose of 10 µM.

2.2.1.8. *In vitro* evaluation of antiproliferative activity in prostate cancers

Prostate cancer is ranked the second cancer in men. Annually, there is 1.6 million new cases and 366,000 deaths because of prostate cancer [55]. In contrast to several androgen dependent prostate cancers, the androgen independent PC3 and DU145 cell lines show an aggressive and invasive behavior [56, 57]. Accordingly, the synthesized hybrid compounds were evaluated at 10 µM concentration against these two cell lines and the results are summarized in Table 8.

Table 8. % Growth inhibition of cell lines representing prostate cancers by compounds **4a**–**u** and imatinib at 10 µM Conc.

Comp	R^1	R^2	X-Y	PC3	DU145	Comp	R^1	R^2	X-Y	PC3	DU145
4a	MeO	H	CONH	28.98	12.22	4l	OH	H	CONH	28.99	34.41
4b	MeO	2-MeO	CONH	43.63	29.65	4m	OH	2-MeO	CONH	25.15	5.49
4c	MeO	3-MeO	CONH	42.88	28.25	4n	OH	3-MeO	CONH	39.29	19.45
4d	MeO	4-MeO	CONH	38.78	26.33	4o	OH	4-MeO	CONH	13.80	3.06
4e	MeO	3,4-CH_2O_2	CONH	26.46	18.67	4p	OH	3,4-CH_2O_2	CONH	27.73	19.58
4f	MeO	3,4,5-TriMeO	CONH	47.57	45.69	4q	OH	3,4,5-TriMeO	CONH	50.19	42.75
4g	MeO	H	NHCO	46.86	14.52	4r	OH	2-MeO	NHCO	10.33	-2.87
4h	MeO	2-MeO	NHCO	70.04	55.06	4s	OH	3-MeO	NHCO	59.64	36.69
4i	MeO	3-MeO	NHCO	48.76	32.65	4t	OH	4-MeO	NHCO	19.59	5.40
4j	MeO	4-MeO	NHCO	68.08	43.54	4u	OH	3,4-CH_2O_2	NHCO	22.55	2.12
4k	MeO	3,4-CH_2O_2	NHCO	111.96	47.07	Imatinib				10.60	14.40

All data are reported as the average of duplicates.

As shown in Fig. 10, imatinib elicited very weak inhibition of both PC3 and DU145 cell lines at the dose of 10 μM concentration. On the other side, almost all tested compounds exerted more pronounced growth inhibition against the employed cell lines. The results showed that compounds **4g**–**k** whose amide linker was configured so that the TMF moiety was attached to the linker's nitrogen atom were much more active relative to compounds **4a**–**e** possessing the opposite linker's configuration, especially in PC3 cell line. Such vivid difference in activity for only different linker configuration confirms the crucial role played by the linker in eliciting the antiproliferative activity, at least, within this cell line. Thus, the 3,4-methylenedioxyphenyl derivative **4k** was lethal to PC3 cell line (111.96% growth inhibition) while the corresponding derivative **4e** with reversed amide configuration showed only 26.46% growth inhibition against the same cell line. Also, the 2-methoxyphenyl derivative **4h** inhibited the growth of PC13 and DU145 cell lines by 70.04 and 55.06% respectively, while compound **4b**, which is the corresponding derivative with the opposite amide linker configuration, showed only 43.63 and 29.65% growth inhibition respectively. In general, as illustrated in Fig. 10, the antiproliferative activity decreased when the TMF moiety was replaced by 6,7-dimethoxy-5-hydroxyflavone moiety. However, such replacement in the corresponding compounds **4f** and **4q** having 3,4,5-trimethoxyphenyl moiety afforded almost the same activity level. Meanwhile, the same replacement in compound **4a** having the unsubstituted-phenyl moiety to afford compound **4l** enhanced the activity against DU145 cells but did not change the activity against PC3. Over almost all tested compounds, DU145 cell line was more susceptible than PC3 cell line to this class of compounds, especially for those bearing amide linker configuration in which TMF moiety was attached to the nitrogen atom of the linker.

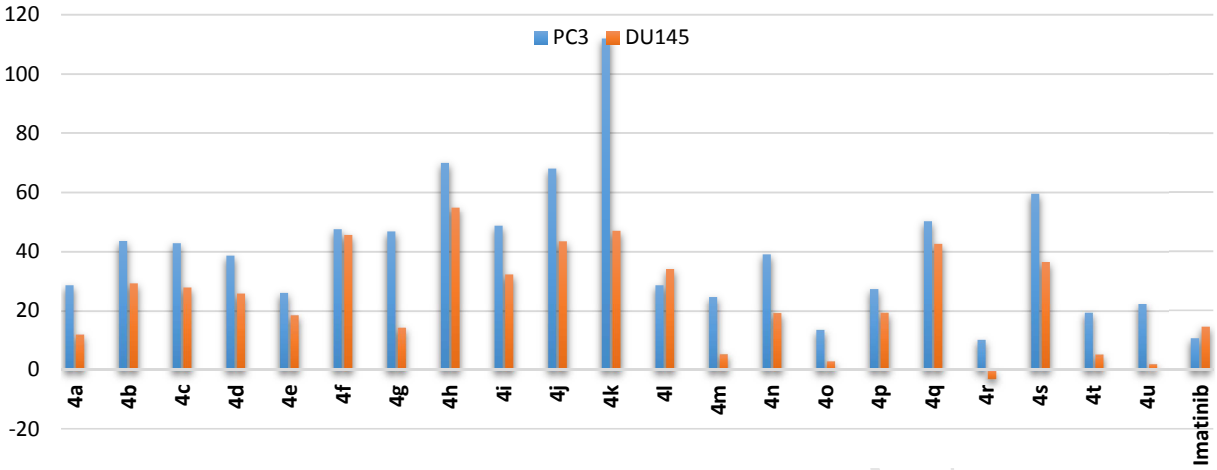


Figure 10. Percentage inhibition of growth inhibition of prostate cancer cells elicited by the synthesized TMS-TMF hybrid compounds and imatinib at a single dose of 10 µM.

2.2.1.9. *In vitro* evaluation of antiproliferative activity in breast cancers

Breast cancer is a leading cause for cancers' mortalities in women aged 20~59 years [48]. Some breast cancers possess amplification of estrogen, progesterone or Her2 receptors, which opens the possibility of targeted therapies through hormonal interference and Her2 inhibitors. Meanwhile, the triple negative breast cancers (TNBC) which are independent on estrogen, progesterone or Her2, are more difficult to treat in the light of absence of targeted therapies [58]. Accordingly, the synthesized hybrid compounds were evaluated by NCI against a panel of breast cancer that included estrogen-dependent MCF7 cells, as well as, TNBC cell lines HS578T, BT549 and MDAMB468. The results are presented in Table 9.

Table 9. % Growth inhibition of cell lines representing breast cancers by compounds **4a**–**u** and imatinib at 10 µM Conc.

Comp	R^1	R^2	X-Y	MCF7	HS578T	BT549	MDAMB468
4a	MeO	H	CONH	7.49	8.00	24.21	9.98
4b	MeO	2-MeO	CONH	28.44	60.01	44.86	24.29
4c	MeO	3-MeO	CONH	12.28	53.79	45.81	21.03
4d	MeO	4-MeO	CONH	21.15	41.58	53.06	24.26
4e	MeO	3,4-CH_2O_2	CONH	17.90	14.04	42.40	1.72
4f	MeO	3,4,5-TriMeO	CONH	65.73	90.80	73.02	52.58
4g	MeO	H	NHCO	22.72	2.76	25.91	15.77

4h	MeO	2-MeO	NHCO	46.59	76.38	54.15	60.77
4i	MeO	3-MeO	NHCO	21.32	43.19	42.53	5.72
4j	MeO	4-MeO	NHCO	33.51	57.06	48.58	24.82
4k	MeO	3,4-CH_2O_2	NHCO	56.36	54.80	58.60	24.52
4l	OH	H	CONH	21.94	88.91	39.91	30.62
4m	OH	2-MeO	CONH	7.54	3.90	10.81	9.38
4n	OH	3-MeO	CONH	13.22	45.52	30.51	45.96
4o	OH	4-MeO	CONH	6.72	43.92	69.01	15.69
4p	OH	3,4-CH_2O_2	CONH	8.35	27.30	15.99	45.38
4q	OH	3,4,5-TriMeO	CONH	50.65	103.91	93.00	40.13
4r	OH	2-MeO	NHCO	-1.88	11.38	25.09	-22.90
4s	OH	3-MeO	NHCO	34.89	34.07	34.25	28.79
4t	OH	4-MeO	NHCO	9.78	25.78	9.60	-6.18
4u	OH	3,4-CH_2O_2	NHCO	11.36	26.07	24.47	17.57
Imatinib				8.10	9.90	-5.00	29.10

All data are reported as the average of duplicates.

At 10 µM dose, imatinib elicited 29.10% growth inhibition against MDAMB468 cell line (Table 9). In addition, it elicited very low inhibition of 9.90% and 8.10 against HS578T and MCF7 cell line. However, it was ineffective against BT549 cell line. As Fig. 11 illustrates, several compounds out of the synthesized and evaluated compounds were much more active relative to imatinib. The results showed an excellent antiproliferative activities against the TNBC cell lines HS578T and BT549 by compounds possessing 3,4,5-trimethoxyphenyl moiety. Thus, compound **4q**, which has 6,7-dimethoxy-5-hydroxyflavone moiety hybridized with the 3,4,5-trimethoxyphenyl moiety, elicited 103.91 and 93.00% inhibition of growth of HS578T and BT549 cell lines respectively, and compound **4f**, which has TMF moiety, showed excellent, but lower inhibition values of 90.80 and 73.02% respectively (Table 9). However, compound **4f** was more active than compound **4q** against the estrogen dependent MCF7 and the TNBC MDAMB468 cell lines, albeit, with moderate activity. In general, as noticed in the previously discussed results, replacement of the TMF moiety of compound **4a** possessing unsubstituted-phenyl moiety and its TMF moiety was attached to the carbonyl group of the amide linker by

6,7-dimethoxy-5-hydroxyflavone moiety in the corresponding compound **4l** results in some enhancement of activity. It is noteworthy that the enhancement of activity of compound **4l** was remarkable against the TNBC HS578T cell line (88.91% relative to only 8.00% for the corresponding compound **4a**). However, similar replacement in compounds **4b**–**e** having similar amide linker configuration but monomethoxyphenyl or 3,4-methylenedioxyphenyl moieties afforded compounds **4m**–**p** possessing in general lower activity. However, reversing the amide linker's configuration of compounds **4b**–**e** resulted in the more active compounds **4h**–**k** as illustrated in Fig. 11. Thus, the 3,4-methylenedioxyphenyl derivative **4k** inhibited the growth of three cell lines by more than 50% and one cell line by 24.52%, while the corresponding compound **4e** which differs only in the configuration of the amide linker inhibited only one cell line by 42.40% and the other cell lines by only 17.90~1.72%. Similarly, the enhanced activity of the 2-methoxyphenyl derivative **4h** was prominent as it inhibited HS578T cell line by 76.38%, MDAMB468 cell line by 60.77% and BT549 cell line by 54.15% while the corresponding compound **4b** with the opposite amide linker configuration inhibited only one cell by more than 50%. Despite the high activity of compounds **4h**–**k** whose TMF moiety was attached to the nitrogen atom of the linker, the corresponding compounds **4r**–**u** retaining the same linker's configuration but having 6,7-dimethoxy-5-hydroxyflavone moiety instead of the TMF moiety was relatively much lower. This cast light on the impact of the substituents of the flavone moiety on the activity.

Collectively, the results identified the TNBC cell lines HS578T as the most responsive among evaluated breast cancer cell lines to this class of hybrid compounds. Thus, HS578T was inhibited by four compounds within the range of 103.91~76.38% and another four compounds within the range of 60.01~53.79%. In addition, the TNBC cell line BT549 elicited significant responses to

the evaluated compounds showing inhibition by two compounds within the range of 93.00~73.02% and four compounds within the range of 69.01~53.06%. Meanwhile, MDAMB468 and MCF7 were the least inhibited cell lines (Fig. 11) eliciting a maximum values for inhibition of growth by 60.77 and 65.73%, respectively.

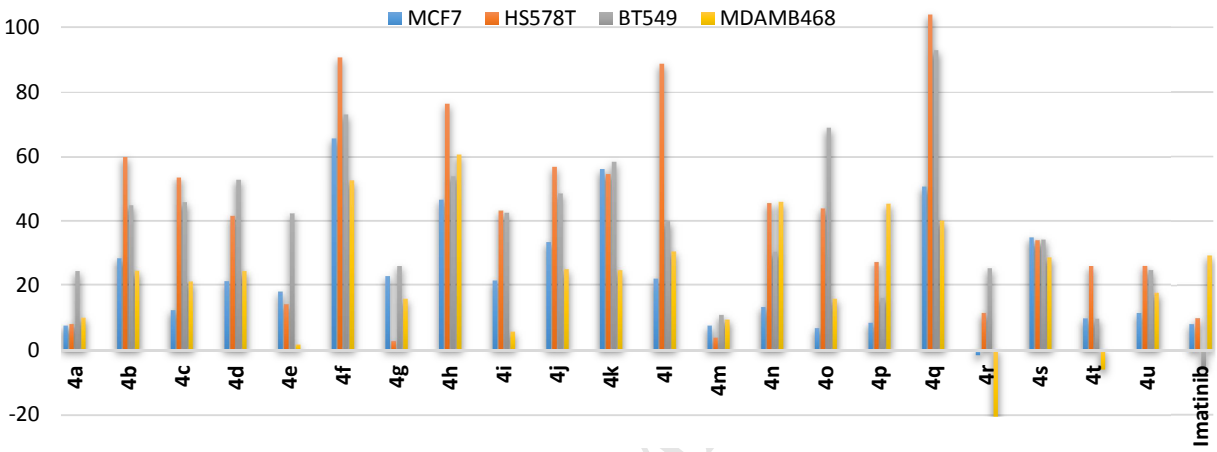


Figure 11. Percentage inhibition of growth inhibition of breast cancer cells elicited by the synthesized TMS-TMF hybrid compounds and imatinib at a single dose of 10 µM.

The calculated average percent values of growth inhibition over all cell lines representing a cancer disease is presented in Table 10 and illustrated in Fig. 12. The results showed that imatinib at 10 µM concentration elicited low inhibition against the employed cell lines representing the evaluated nine cancer diseases. In comparison, the results showed an evident high susceptibility of hematologic cancers to this class of compounds as eight compounds out of the evaluated twenty-one compounds elicited growth inhibition of the employed leukemic cell lines by more than 50%. Five compounds out of these eight compounds possessed TMF moiety and amide linker configuration in which the nitrogen atom of the linker was attached to the TMF moiety indicating high probability for this combined molecular features to produce antileukemic agents. The 3,4-methylenediophenyl derivative **4k** was the most active compound among them

eliciting an average growth inhibition value of 99.88% over the employed cell lines representing leukemia. However, the 3,4,5-trimethoxylphenyl derivative **4f** whose amide linker configuration was the opposite to compound **4k** but retained the TMF moiety came in the second rank eliciting an average growth inhibition value of 77.43% over the employed cell lines representing leukemia. As table 10 shows, four compounds; the 3,4,5-trimethoxyphenyl derivative **4q** and **4f** possessing amide linker configuration in which the flavone moiety was attached to the carbonyl group of the linker, as well as, 2-methoxyphenyl derivative **4h** and 3,4-methylenedioxyphenyl derivative **4k** possessing the opposite linker's configuration, elicited an average nearby values over cell lines representing NSCLC. Also, the three compounds **4f**, **4h** and **4k** which were highly active against cell lines representing hematologic cancers and NSCLC were the best inhibitors of the growth of cell lines representing colon cancers within a range of 54.60~49.78% at 10 µM concentration. However, compound **4l**, which have an unsubstituted-phenyl moiety and a 6,7-dimethoxy-5-hydroxyflavone moiety attached to the carbonyl group of the linker, was ranked the first against cell lines representing CNS cancers. Meanwhile, the 3,4,5-trimethoxyphenyl derivative **4q**, which have also 6,7-dimethoxy-5-hydroxyflavone moiety and similar amide linker configuration as well as the 3,4-methylenedioxyphenyl derivative **4k** bearing TMF moiety and the opposite amide linker configuration came in the second rank with almost similar average inhibition percent values. When considering the activity against cell lines representing melanoma and renal cancers, the three compounds **4f**, **4h** and **4k** came again to the fore in the first, second and third rank respectively. However, for ovarian cancer, compound **4q** ranked the best growth inhibitor followed by compounds **4l**, **4h** and **4f**, which elicited nearby values for the elicited average growth inhibition. Considering the average growth inhibition values over prostate cancer cell lines, the three compounds **4k**, **4h** and **4j** came in the first, second and third rank respectively.

In the case of cell lines representing breast cancers, the two compounds **4q** and **4f** came in the first and the second rank with very similar average inhibition percent values, while the two compounds **4h** and **4k** came in the third and the forth rank. As shown in Fig. 12, the collective results showed that the three compounds **4f**, **4h** and **4k** possessed promising antiproliferative activity by more than 50% average inhibition against several cell lines representing multiple cancer diseases. In addition, compound **4q** elicited also a promising antiproliferative activity nearby but less than 50% average inhibition against several cell lines representing multiple cancers.

Table 10. Average percent inhibition values of growth over cell lines representing certain cancer disease by 10 µM dose of compounds **4a**–**u** and imatinib.

Compound	Leukemia	NSCLC	Colon Cancer	CNS Cancer	Melanoma	Ovarian Cancer	Renal Cancer	Prostate Cancer	Breast Cancer
4a	35.99	16.36	11.17	10.37	18.59	17.16	16.74	20.60	12.42
4b	42.41	39.02	27.76	26.11	29.38	33.74	38.55	36.64	39.40
4c	57.19	40.32	33.48	25.71	27.08	35.94	39.04	35.57	33.23
4d	44.15	31.33	33.01	21.88	32.02	36.39	41.87	32.56	35.01
4e	43.22	21.73	18.14	7.12	22.65	24.17	23.20	22.57	25.50
4f	77.43	56.58	49.78	43.24	61.99	44.09	54.50	46.63	70.53
4g	58.63	29.89	23.13	21.61	23.88	20.03	25.73	30.69	16.79
4h	70.87	55.10	54.60	44.04	54.84	44.76	53.97	62.55	59.47
4i	53.88	39.08	40.49	36.25	28.50	32.94	36.98	40.71	28.19
4j	72.75	48.39	40.35	35.18	36.83	33.95	47.70	55.81	40.99
4k	99.88	54.58	52.36	49.25	46.70	35.14	52.52	79.52	50.62
4l	5.18	46.75	24.67	59.55	30.22	45.96	44.26	31.70	45.35
4m	27.70	12.10	4.13	8.12	9.52	10.00	13.94	15.32	7.91
4n	32.43	33.59	20.95	17.26	21.07	27.90	24.74	29.37	33.80
4o	3.09	21.02	4.44	42.66	11.93	15.56	15.00	8.43	33.84
4p	9.65	26.92	16.97	24.64	14.63	35.35	21.80	23.66	26.57
4q	42.53	59.96	38.82	49.66	43.63	49.57	43.95	46.47	71.92
4r	11.09	13.26	2.40	16.53	8.39	1.87	4.78	3.73	2.92
4s	52.91	38.26	30.10	24.00	34.83	32.85	27.40	48.17	33.00
4t	17.40	25.58	8.56	24.24	12.74	9.23	17.33	12.50	9.75
4u	16.02	27.27	6.99	39.57	14.38	19.36	22.04	12.34	21.70
Imatinib	9.00	15.68	5.34	5.80	4.10	-7.32	1.87	12.50	10.53

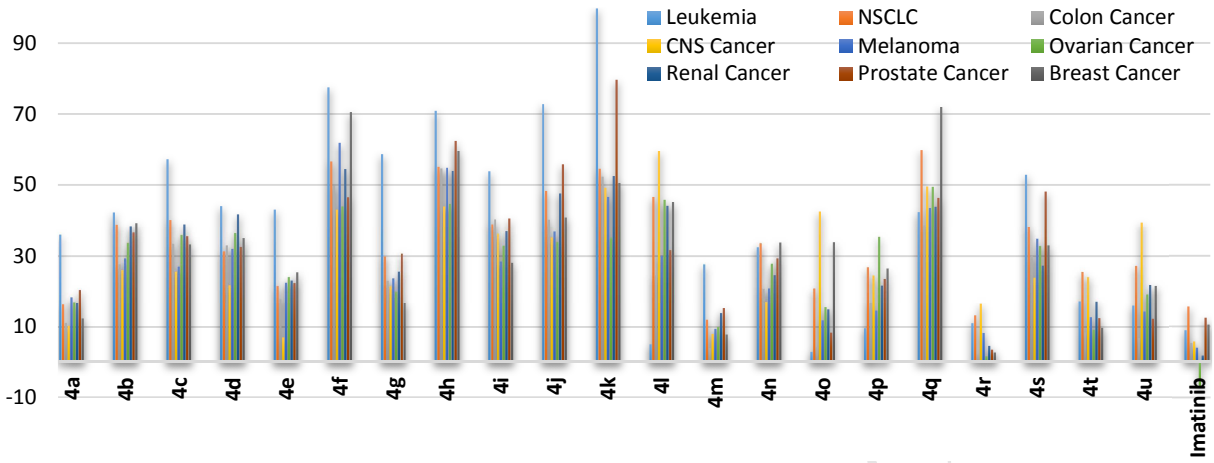


Figure 12. Average Percentage inhibition values of growth over cell lines representing several cancer disease by the synthesized TMS-TMF hybrid compounds and imatinib at a single dose of 10 µM.

2.3. Cytotoxicity against normal human cell lines

For a successful anticancer agent, it should safe eliciting a selective cytotoxic activity against cancer cells while the normal cells should not be inhibited. Therefore, we determined IC_{50} values for the most active compounds **4f**, **4h**, **4k** and **4q** on the cells viability of two normal human cell lines; L132 (human lung epithelial cell) and IOSE-80PC (human ovarian epithelial cell) using MTT cells viability assay. As shown in Table 11, the measured IC_{50} values for compounds **4f**, **4h** and **4q** against the normal human lung epithelial cell line L132 were above 100 µM while compound **4k** elicited IC_{50} value of 55.04 µM. In addition, the measured IC_{50} values for compounds **4h**, **4k** and **4q** against the normal human ovarian epithelial cell line IOSE-80PC were above 100 µM while compound **4f** elicited IC_{50} value of 67.61 µM. Considering that the cytotoxic effects against cancer cells were measured for these compounds at the much lower concentration of 10 µM, it might be concluded that these compounds elicit selective cytotoxic effects against cancer cells rather than normal cells.

Table 11. Cytotoxicity against normal human cell lines

Compound	IC_{50} (μM)	
	L132[a]	IOSE-80PC[b]
4f	>100	67.61
4h	>100	>100
4k	55.04	>100
4q	>100	>100

[a] Normal human lung epithelial cell line.
[b] Normal human ovarian epithelial cell line.

2.4. *In vitro* investigation of the induced cell death pathway

Among the synthesized and evaluated TMS-TMF hybrid analogs, compound **4f**, which possessed a promising antiproliferative activity against several cell lines representing multiple cancers, was selected for further study of the mechanism leading to the induced cell death. Because none of the cell lines used in the nine panels was available to us to conduct the mechanistic study at our institute, the available human cervical cancer cells HeLa was used to perform this study.

As HeLa cells was not included in the cancer cells panel of NCI, we have first determined the IC_{50} for inhibition of growth of HeLa cells by compound **4f** by MTT viability assay. The assay showed that the IC_{50} value of compound **4f** is 12.07 μM. Next, we examined the morphological changes of the nuclei in HeLa cells in presence of 10 μM concentration of compound **4f** after 48 hours using DAPI (4',6-diamidino-2-phenylindole) staining. Because of the preferential passage of DAPI through damaged biological membranes of dead cells over intact membranes of living cells, as well as, its tight binding with adenine–thymine rich regions in DNA and emission of blue fluorescence upon excitation by 358 nm UV light, it yields information about the cells' nuclei including shape, size and density. As fluorescence microscopy images shown in Fig. 13.

illustrate, HeLa cells treated with compound **4f** emit a highly bright blue color from the majority of cells indicating presence of many apoptotic bodies, while the untreated control living cells show less bright blue color indicating much less number of apoptotic cells. This difference in fluorescence arises from the fact that chromatin in the nuclei of apoptotic cells converts into fragmented, condensed and packed small apoptotic bodies, which would produce higher intensity of DAPI stain. On the opposite, chromatin of living cells exists as a less packed network, so it will show less intensity of DAPI stain.

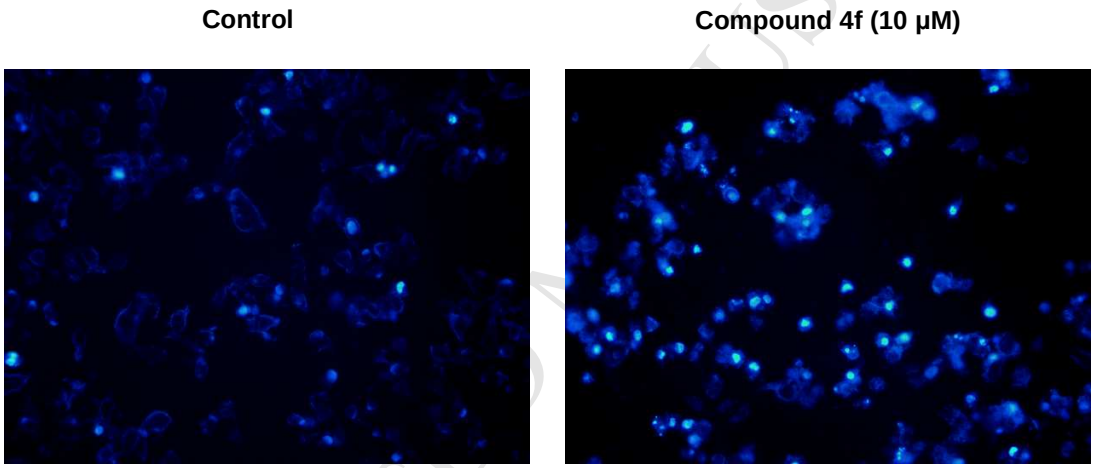


Figure 13. Morphological changes in HeLa Cells induced by 10 µM concentration of compound **4f** after 48 hours

Next, we analyzed the DNA contents and cell cycle distribution of HeLa cells in presence of increasing concentrations of compound **4f**, as well as, at increasing time intervals using flow cytometry after propidium iodide (PI) staining. In principle, cells at G_1 phase have one copy of DNA, therefore will produce one fold fluorescence. While cells at G_2/M phase possess two copies of DNA, and hence, will produce two folds fluorescence. Because DNA is undergoing synthesis at S phase, the fluorescence value of these cells will be between those of G_1 and G_2/M cells. In contrast, DNA fragmentation that might arise from apoptosis will result in loss of DNA from these cells upon aqueous solution staining. Accordingly, lower fluorescence values

identified as sub-G_1 will be proportional to the populations of these cells with fragmented DNA content. As Fig. 14 illustrates, the fluorescence signal in the sub-G_1 region increased in dose dependent manner. Thus, upon increasing the concentration of compound **4f** from zero to 10 µM concentration the fluorescence signal increased from 6.24% to 19.14%. This indicated dose dependent cell death triggered by compound **4f** that might be an apoptotic cell death. Similarly, a time dependent increase of the fluorescence signal was found in the sub-G_1 region from 4.43% at zero time to 26.61% after 48h. Again, this indicated time dependent cell death triggered by compound **4f** that might be an apoptotic cell death.

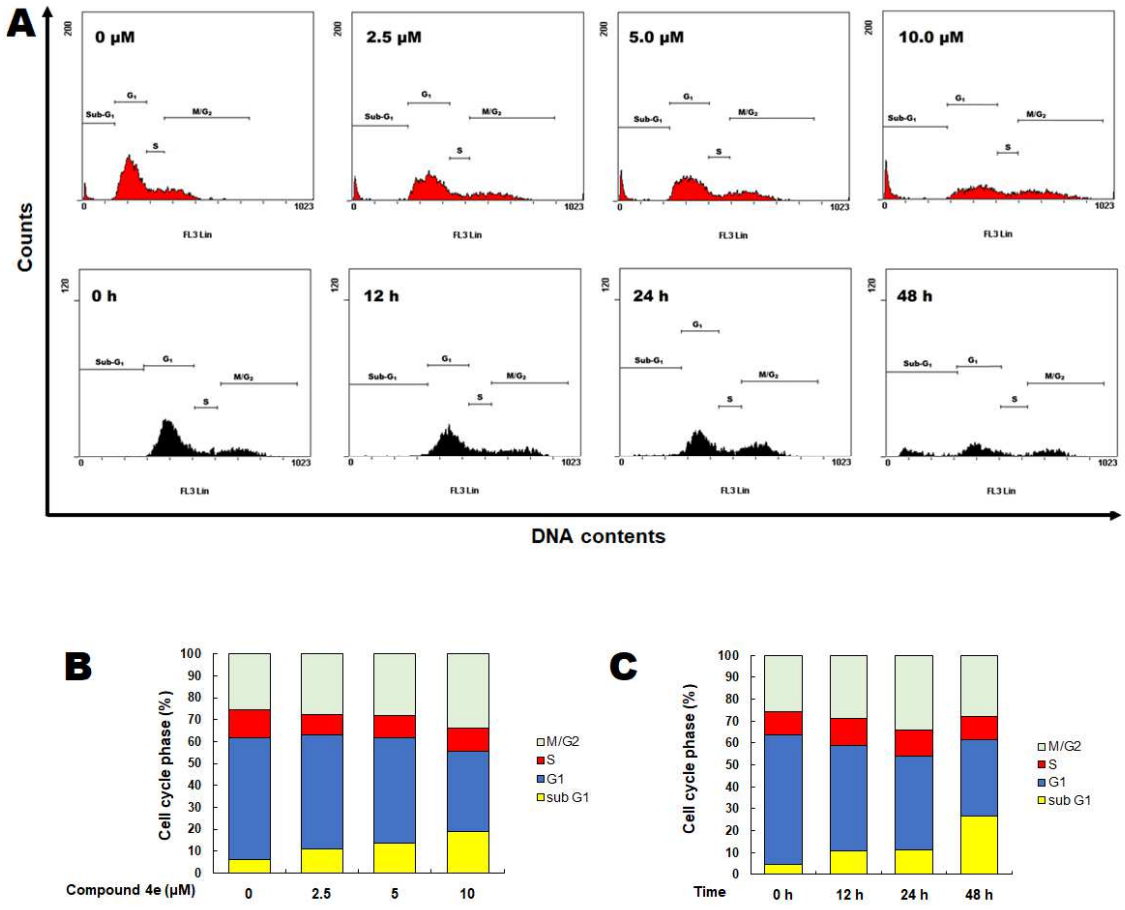


Figure 14. Flow cytometric analysis of dose and time-dependent effects on Hela cell cycle by compound **4f**: (a) Cell cycle distribution histograms of HeLa cells treated with compound **4f** at different concentrations and different time intervals as revealed from flow cytometry after PI staining; (b) Percentage of the distribution of cell cycle phases of

HeLa cells treated with compound **4f** at different concentrations; (c) Percentage of the distribution of cell cycle phases of HeLa cells treated with compound **4f** at time intervals.

Because the increase in sub-G_1 region could be because of apoptotic or necrotic cells, we further investigated the effect of compound **4f** on HeLa cells over increasing time and concentrations using fluorescence-activated cell sorting (FACS) after staining cells with fluorescently labeled annexin V and propidium iodide. As illustrated in Fig. 15, there was a steady dose and time dependent increase of the percent of early apoptotic cells (annexin positive/propidium iodide negative) and late apoptotic cells (annexin positive/propidium iodide positive). Accordingly, the above mentioned detected increase in fluorescence in sub-G1 region might be attributed to apoptotic cell death.

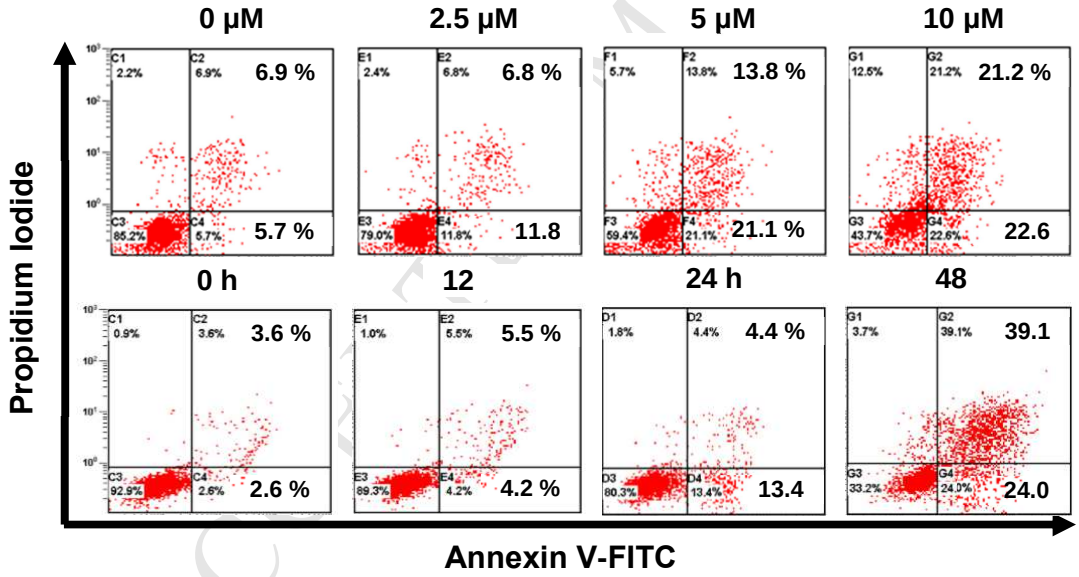


Figure 15. Histograms of fluorescence-activated cell sorting after staining with annexin V-FITC and propidium iodide over increasing concentrations of compound **4f** and increasing incubation time.

3. Conclusion

The relatively higher success rate in drug discovery projects upon using the privileged natural products structures stimulated us to look for privileged cytotoxic natural product scaffolds as

starting points to develop new chemical entities in search for novel promising anticancer lead compounds. The natural product 3,5,4'-trimethoxystilbene was suggested as a promising cytotoxic molecule. However, *in vivo cis*/*trans* isomerization of TMS imposes doubts about its usefulness. Meanwhile, TMF possess a modest cytotoxic potency. In our rational, we anticipated a chimeric hybrid molecules between TMS and TMF analogs in which an amide linker replaces the problematic olefinic moiety of TMS might provide promising anticancer lead compounds. The two possible configurations for the amide linker were planned to be explored in the designed compounds. The synthesis was conducted straightforwardly in good yields following a sequence of well-established reactions. To get a relatively comprehensive evaluation of the antiproliferative activity of the prepared hybrid compounds, the activity was assessed *in vitro* by NCI against nine panels representing major cancer diseases. In addition to the influence of substitution pattern of the phenyl moiety and the switch between TMF and 6,7-dimethoxy-5-hydroxyflavone moieties on the activity, the results revealed a crucial impact of the amide linker configuration on the elicited biological activity. Out of the evaluated twenty-one compounds, the collective results presented four compounds; **4f**, **4h**, **4k** and **4q** as promising anticancer compounds eliciting high growth inhibition at 10 µM concentration against several cell lines representing multiple cancers diseases. Evaluation of the cytotoxic effects of these promising compounds on normal human cell lines suggests that they might exert a selective inhibition of growth of cancer cells. These the compounds might serve as leads for further development of novel anticancer agents. Among them, compound **4f** was selected for investigation of the induced cell death in human cervical cancer HeLa cells using fluorescence microscopy after DAPI staining, flow cytometry after propidium iodide staining and FACS analysis after annexin

V and propidium iodide staining. The mechanistic studies showed that compound **4f** triggers cell death through induction of apoptosis in human cervical cancer HeLa cells.

4. Experimental

4.1.Chemistry

4.1.1. General

NMR spectral analyses were acquired using Brucker Avance 400 spectrometer (400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR) or Agilent 500 spectrometer (500 MHz for ^{1}H NMR, 125 MHz for ^{13}C NMR). High-resolution spectra were performed on Jeol accuTOF (JMS-T100TD) equipped with a DART ion source from Ionsense, Tokyo, Japan in the positive modes. Analytical TLC was carried out using precoated silica gel (E. Merck Kiesegel 60F_{254}, layer thickness 0.25 mm) and flash column chromatography was performed with using Merck Kiesegel 60 Art 9385 (230–400 mesh). Solvents, chemicals and reagents were purchased from Sigma-Aldrich or Tokyo chemical industry (TCI), and used without further purification.

4.1.2. 2-Acetyl-3,4,5-trimethoxyphenol (6)

Boron trifluoride diethyletherate (21.5 mL, 81.45 mmol) was added to a mixture of 3,4,5-trimethoxyphenol (**5**, 3.0 g, 16.29 mmol) and acetic anhydride (8.25 mL). The reaction mixture was refluxed overnight, and then cooled to 0 °C. The resulting precipitate was filtered. The filtrate was poured into aqueous solution of ethanolamine. The mixture was stirred for 1 h and then extracted with EtOAc. The extract was washed with brine, dried over anhydrous magnesium sulfate, and filtered. The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography to give the titled compound **6**. Yield 60%. ^{1}H

NMR ($CDCl_3$, 400 MHz) δ 13.44 (1H, s), 6.23 (1H, s), 4.00 (3H, s), 3.89 (3H, s), 3.78 (3H, s), 2.65 (3H, s). Reported compound [41].

4.1.3. General procedure for (*E*)-1-(6-Hydroxy-2,3,4-trimethoxyphenyl)-3-(3-substituted-phenyl)prop-2-en-1-one (7)

Sodium methoxide (1.31 g, 24.22 mmol) was added to a stirred solution of compound **6** (1.49 g, 9.08 mmol) and the appropriate benzaldehyde derivative (6.06 mmol) in methanol (25 mL) stirred overnight at room temperature. The reaction mixture was neutralized with 6 N HCl and the formed precipitate was filtered, washed with distilled water, and purified by silica gel column chromatography to give the titled compound **7**

(*E*)-Methyl 3-(3-(6-hydroxy-2,3,4-trimethoxyphenyl)-3-oxoprop-1-enyl)benxoate (7a)

Yield 59%; solid; mp: 119.1 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 13.60 (1H, s), 8.31 (1H, br s), 8.06 (1H, d, J = 7.7 Hz), 8.00 (1H, d, J = 15.7 Hz), 7.83 (1H, d, J = 15.7 Hz), 7.81 (1H, d, J = 7.7 Hz), 7.50 (1H, t, J = 7.7 Hz), 6.31 (1H, s), 3.96 (3H, s), 3.95 (3H, s), 3.92 (3H, s), 3.85 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 177.1, 166.3, 160.1, 158.1, 154.6, 152.6, 140.6, 132.1, 132.0, 131.1, 130.1, 129.2, 127.2, 112.9, 108.8, 96.4, 62.2, 61.6, 56.5, 52.6.

(*E*)-1-(6-Hydroxy-2,3,4-trimethoxyphenyl)-3-(3-nitrophenyl)prop-2-en-1-one (7b)

Yield 59%; solid; mp: 133.5 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.95 (1H, s), 8.55 (1H, s), 8.27 (1H, d, J = 8.2 Hz), 8.23 (1H, d, J = 7.8 Hz), 7.74 (1H, t, J = 8.1 Hz), 7.70 (2H, d, J = 3.8 Hz), 6.41 (1H, s), 3.85 (3H, s), 3.84 (3H, s), 3.71 (3H, s); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 192.9, 158.8, 158.2, 153.6, 148.8, 140.4, 137.0, 135.1, 134.5, 131.0, 130.5, 125.0, 123.5, 110.9, 96.9, 62.1, 61.1, 56.6.

4.1.4. General procedure for cyclization of chalcones to flavones

A mixture of compound **7** (4.43 mmol), dimethyl sulfoxide (15 mL), and iodine (0.11 g, 0.44 mmol) was heated at 110 °C. After reaction completion, the mixture was cooled to room temperature, poured into sodium thiosulfate solution, and extracted with dichloromethane. The organic layer was dried over anhydrous sodium sulfate, concentrated, and recrystallized from dichloromethane/*n*-hexane to give the titled compound **8**.

Methyl 3-(5,6,7-trimethoxy-4-oxo-4*H*-chromen-2-yl)benzoate (8a)

Yield 88%; solid; mp: 169.3 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.59 (1H, t, J = 1.6 Hz), 8.19 (1H, m), 8.05 (1H, m), 7.60 (1H, t, J = 7.8 Hz), 6.87 (1H, s), 6.73 (1H, s), 4.01 (3H, s), 4.00 (3H, s), 3.99 (3H, s), 3.93 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 177.1, 166.3, 160.0, 158.0, 154.6, 152.6, 140.6, 132.1, 132.0, 131.1, 130.1, 129.2, 127.2, 112.9, 108.8, 96.4, 62.2, 61.6, 56.4, 52.5.

5,6,7-Trimethoxy-2-(3-nitrophenyl)-4*H*-chromen-4-one (8b)

Yield 88%; solid; mp: 186.5 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.80 (1H, t, J = 1.8 Hz), 8.53 (1H, dd, J_1 = 7.7 Hz, J_2 = 0.8 Hz), 8.41 (1H, m), 7.85 (1H, t, J = 8.0 Hz), 7.33 (1H, s), 7.05 (1H, s), 3.98 (3H, s), 3.81 (3H, s), 3.78 (3H, s); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 176.7, 158.2 (2C), 154.4, 152.6, 148.8, 140.7, 133.5, 131.4, 130.2, 125.6, 120.9, 113.0, 109.6, 96.3, 62.2, 61.6, 56.5.

4.1.5. 3-(5,6,7-Trimethoxy-4-oxo-4*H*-chromen-2-yl)benzoic acid (9a)

To a solution of compound **8a** (164 mg, 0.44 mmol) in methanol was added potassium hydroxide (123 mg, 2.20 mmol) and the mixture was refluxed overnight. After reaction completion, the mixture was cooled to rt, neutralized with 1 N HCl, and extracted with ethyl acetate. The extract was washed with brine, dried over anhydrous sodium sulfate, and filtered. The solvent was removed under reduced pressure to give the titled compound **9a**. Yield 97%; solid; mp: 247.8 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.53 (1H, br s), 8.31 (1H, d, J = 7.8 Hz), 8.13 (1H, d, J = 7.8

Hz), 7.70 (1H, t, *J* = 7.8 Hz), 7.26 (1H, s), 6.88 (1H, s), 3.98 (3H, s), 3.82 (3H, s), 3.78 (3H, s); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 175.6, 166.7, 159.3, 157.7, 154.0, 151.5, 139.9, 132.0, 131.7, 131.4, 130.3, 129.5, 126.3, 112.1, 108.2, 97.4, 61.8, 61.0, 56.5.

4.1.6. 2-(3-Aminophenyl)-5,6,7-trimethoxy-4*H*-chromen-4-one (9b)

To a solution of compound **8b** (2.8 g, 5.58 mmol) in in ethanol (100 mL) was added tin(II) chloride dehydrate (3.78 g, 16.75 mmol). The mixture was refluxed for 2.5 hours. After completion of reaction, the reaction was cooled and water was added. A saturated sodium bicarbonate solution was added until pH 9. The mixture was extracted with ethyl acetate, washed with brine, dried over anhydrous sodium sulfate and evaporated under reduced pressure. Column chromatography purification (silica gel, CH_2Cl_2: MeOH = 80: 1) afforded the titled compound **9b**. Yield 87%; solid; mp: 166.3 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27 (2H, m), 7.17 (1H, s), 6.82 (1H, dt, J_1 = 6.8 Hz, J_2 = 2.2 Hz), 6.80 (1H, s), 6.61 (1H, s), 3.99 (3H, s), 3.98 (3H, s), 3.92 (3H, s) ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.3, 161.4, 157.7, 154.6, 152.5, 146.9, 140.3, 132.6, 129.9, 117.8, 116.2, 113.0, 112.0, 108.4, 96.3, 62.2, 61.6, 56.3.

4.1.7. General procedure for preparation of *N*-[(substituted)phenyl]-3-(5,6,7-trimethoxy-4-oxo-4*H*-chromen-2-yl)benzamides (4a–f)

Iodine (160 mg, 0.63 mmol, 1.5 equivalents) was added to a stirred solution of trimethyl phosphite (0.07 mL, 0.63 mmol, 1.5 equivalents) in dichloromethane (4 mL) at 0 °C. After all solid iodine was completely dissolved, compound **9a** (150 mg, 0.42 mmol, 1 equivalent) was added followed by triethylamine (0.29 mL, 2.11 mmol, 5.0 equivalents). The reaction mixture was stirred for 10 min at 0 °C before adding the appropriate primary amine derivative (0.84 mmol, 2 equivalents) and the mixture was stirred for 10 min at 0 °C. The reaction was removed from cooling bath and stirred for further 3 h at room temperature. The mixture was diluted with

saturated aqueous sodium bicarbonate solution and extracted with dichloromethane. The organic layer was sequentially washed with water, 1 N HCl, water, and brine then dried over anhydrous sodium sulfate. After filtration, the solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography. The compound was further purified by recrystallization from dichloromethane/*n*-hexane to give the titled compounds **4a–f**.

***N*-Phenyl-3-(5,6,7-trimethoxy-4-oxo-4*H*-chromen-2-yl)benzamide (4a)**

Yield 73%; solid; mp: 180.3 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.73 (1H, s), 8.31 (1H, br s), 8.00 (1H, d, J = 7.8 Hz), 7.97 (2H, d, J = 7.8 Hz), 7.64 (1H, d, J = 7.8 Hz), 7.42 (1H, t, J = 7.8 Hz), 7.39 (2H, t, J = 7.8 Hz), 7.16 (1H, t, J = 7.8 Hz), 6.67 (1H, s), 6.45 (1H, s), 3.92 (3H, s), 3.83 (3H, s), 3.76 (3H, s); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.6, 166.2, 160.6, 158.3, 154.5, 152.2, 140.4, 139.1, 137.2, 131.5, 130.9, 129.4, 129.2 (2C), 128.3, 124.9, 124.6, 120.6 (2C), 112.7, 108.6, 96.4, 62.2, 61.6, 56.7; HRMS: *m/z* calculated for $C_{25}H_{22}NO_6$: 432.1442 $[M+H]^+$. Found 432.1386.

***N*-(2-methoxyphenyl)-3-(5,6,7-trimethoxy-4-oxo-4*H*-chromen-2-yl)benzamide (4b)**

Yield 57%; Solid; mp: 154.9 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.61 (1H, s), 8.53 (1H, d, J = 7.7 Hz), 8.48 (1H, br s), 8.00 (1H, d, J = 7.8 Hz), 7.96 (1H, d, J = 7.7 Hz), 7.63 (1H, t, J = 7.7 Hz), 7.12 (1H, t, J = 7.8 Hz), 7.04 (1H, t, J = 7.8 Hz), 6.95 (1H, d, J = 7.8 Hz), 6.87 (1H, s), 6.73 (1H, s), 4.00 (3H, s), 3.99 (3H, s), 3.95 (3H, s), 3.93 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 177.1, 164.3, 160.0, 158.1, 154.5, 152.5, 148.2, 140.6, 136.2, 132.4, 129.4, 129.0, 128.9, 127.4, 125.3, 124.4, 121.2, 120.0, 112.9, 110.0, 108.8, 96.5, 62.2, 61.6, 56.5, 55.9; HRMS: *m/z* calculated for $C_{26}H_{24}NO_7$: 462.1547 $[M+H]^+$. Found 462.1584.

***N*-(3-methoxyphenyl)-3-(5,6,7-trimethoxy-4-oxo-4*H*-chromen-2-yl)benzamide (4c)**

Yield 76%; solid; mp: 164.5 °C; 1H NMR (CDCl3, 400 MHz) δ 9.70 (1H, s), 8.32 (1H, br s), 8.00 (1H, d, J = 7.8 Hz), 7.71 (1H, br s), 7.66 (1H, d, J = 7.8 Hz), 7.48 (1H, d, J = 8.1 Hz), 7.43 (1H, t, J = 7.8 Hz), 7.27 (1H, t, J = 8.1 Hz), 6.71 (1H, m), 6.68 (1H, s), 6.48 (1H, s), 3.92 (3H, s), 3.82 (6H, s), 3.75 (3H, s); ^{13}C NMR (CDCl3, 100 MHz) δ 177.4, 165.9, 160.3, 160.1, 158.1, 154.3, 151.9, 140.1, 140.0, 136.8, 131.2, 130.7, 129.6, 129.1, 128.1, 124.6, 112.6, 112.4, 110.2, 108.4, 106.0, 96.2, 62.0, 61.4, 56.4, 55.3; HRMS: *m/z* calculated for $C_{26}H_{24}NO_7$: 462.1547 $[M+H]^+$. Found 462.1521.

***N*-(4-methoxyphenyl)-3-(5,6,7-trimethoxy-4-oxo-4*H*-chromen-2-yl)benzamide (4d)**

Yield 80%; solid; mp: 161.4 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.46 (1H, s), 8.31 (1H, br s), 7.99 (1H, d, *J* = 7.8 Hz), 7.83 (2H, d, *J* = 8.8 Hz), 7.67 (1H, d, *J* = 7.8 Hz), 7.43 (1H, t, *J* = 7.8 Hz), 6.91 (2H, d, *J* = 8.8 Hz), 6.68 (1H, s), 6.46 (1H, s), 3.92 (3H, s), 3.84 (3H, s), 3.82 (3H, s), 3.78 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 177.3, 165.6, 160.4, 158.1, 156.4, 154.3, 152.0, 140.1, 136.9, 132.0, 131.3, 130.6, 129.1, 128.0, 124.6, 122.0 (2C), 114.1 (2C), 112.5, 108.4, 96.2, 62.0, 61.4, 56.4, 55.5; HRMS: *m/z* calculated for $C_{26}H_{24}NO_7$: 462.1547 $[M+H]^+$. Found 462.1535.

***N*-(3,4-methylenedioxyphenyl)-3-(5,6,7-trimethoxy-4-oxo-4*H*-chromen-2-yl)benzamide (4e)**

Yield 73%; solid; mp: 218.0 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.51 (1H, s), 8.30 (1H, br s), 7.99 (1H, d, *J* = 7.8 Hz), 7.88 (1H, d, *J* = 7.8 Hz), 7.61 (1H, br s), 7.45 (1H, t, *J* = 7.8 Hz), 7.31 (1H, dd, *J* = 8.4, 1.6 Hz), 6.80 (1H, d, *J* = 8.4 Hz), 6.69 (1H, s), 6.46 (1H, s), 5.97 (2H, s), 3.93 (3H, s), 3.83 (3H, s), 3.77 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 177.4, 165.6, 160.4, 158.1, 154.3, 152.0, 147.8, 144.2, 140.2, 136.8, 133.1, 131.3, 130.6, 129.2, 128.1, 124.6, 113.5, 112.5, 108.4, 108.1, 103.0, 101.2, 96.2, 62.0, 61.4, 56.4.

3-(5,6,7-trimethoxy-4-oxo-4*H*-chromen-2-yl)-*N*-(3,4,5-trimethoxyphenyl)benzamide (4f)

Yield 77%; solid; mp: 136.9 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.40 (1H, s), 8.38 (1H, br s), 8.04 (1H, d, J = 7.9 Hz), 7.78 (1H, d, J = 7.9 Hz), 7.51 (1H, t, J = 7.9 Hz), 7.29 (2H, s), 6.72 (1H, s), 6.59 (1H, s), 3.95 (3H, s), 3.87 (6H, s), 3.85 (3H, s), 3.84 (3H, s), 3.77 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 177.4, 165.7, 160.5, 158.2, 154.4, 153.3 (2C), 152.1, 140.2, 136.8, 134.9, 134.7, 131.4, 130.6, 129.3, 128.3, 124.6, 112.5, 108.6, 98.0 (2C), 96.2, 61.9, 61.4, 61.0, 56.4, 56.1 (2C); HRMS: *m/z* calculated for $C_{28}H_{28}NO_9$: 522.1759 $[M+H]^+$. Found 522.1754.

4.1.8. General procedure for *N*-(3-(5,6,7-trimethoxy-4-oxo-4*H*-chromen-2-yl)phenyl)benzamide derivatives 4g–k

To a solution of compound **9b** (100 mg, 0.31 mmol) was added potassium carbonate (211.1 mg, 1.53 mmol). After stirring at room temperature for 5 min, the appropriate benzoyl chloride derivative (0.37 mmol, 1.2 equivalents) and stirring was continued. After consumption of starting material, the reaction was quenched with water, extracted with dichloromethane, washed with brine, dried over anhydrous sodium sulfate then evaporated under reduced pressure. Recrystallization afforded the titled compounds **4g–k**

***N*-(3-(5,6,7-Trimethoxy-4-oxo-4*H*-chromen-2-yl)phenyl)benzamide (4g)**

Yield 92%; solid; mp: 191.8 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.46 (1H, s), 8.40 (1H, s), 8.05–7.98 (3H, m), 7.80 (1H, d, J = 7.9 Hz), 7.65–7.61 (1H, m), 7.60–7.53 (3H, m), 7.14 (1H, s), 6.72 (1H, s), 3.97 (3H, s), 3.82 (3H, s), 3.78 (3H, s); ^{13}C NMR (125 MHz, DMSO-d_6) δ 176.3, 166.4, 160.9, 158.3, 154.7, 152.3, 140.6, 140.5, 135.3, 132.5, 132.0, 130.2, 129.2, 128.4, 124.0, 122.1, 118.4, 112.9, 108.4, 97.8, 62.6, 61.7, 57.2; HRMS: *m/z* calculated for $C_{25}H_{22}NO_6$: 432.1447 $[M+H]^+$. Found 432.1483.

2-Methoxy-*N*-(3-(5,6,7-trimethoxy-4-oxo-4*H*-chromen-2-yl)phenyl)benzamide (4h)

Yield 84%; solid; mp: 122.3 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.32 (1H, s), 8.37 (1H, s), 7.97 (1H, d, J = 8.2 Hz), 7.78 (1H, d, J = 7.9 Hz), 7.68 (1H, dd, J_1= 7.5 Hz, J_2= 1.6 Hz), 7.57–7.51 (2H, m), 7.21 (1H, d, J = 8.4 Hz), 7.14 (1H, s), 7.09 (1H, t, J = 8.3 Hz), 6.72 (1H, s), 3.97 (3H, s), 3.93 (3H, s), 3.82 (3H, s), 3.78 (3H, s); ^{13}C NMR (125 MHz, DMSO-d_6) δ 176.3, 165.6, 160.9, 158.3, 157.2, 154.7, 152.3, 140.6, 140.4, 133.0, 132.1, 130.4, 130.3, 125.3, 123.4, 121.9, 121.2, 117.7, 112.9, 112.7, 108.5, 97.9, 62.6, 61.7, 57.2, 56.6; HRMS: *m/z* calculated for $C_{26}H_{24}NO_7$: 462.1553 $[M+H]^+$. Found 462.1584.

3-Methoxy-*N*-(3-(5,6,7-trimethoxy-4-oxo-4*H*-chromen-2-yl)phenyl)benzamide (4i)

Yield 89%; solid; mp: 159.7 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.43 (1H, s), 8.39 (1H, s), 8.02 (1H, dt, J_1 = 8.2 Hz, J_2 = 0.8 Hz), 7.80 (1H, d, J = 7.8 Hz), 7.61–7.52 (3H, m), 7.48 (1H, t, J = 8.0 Hz), 7.19 (1H, dd, J_1 = 8.2 Hz, J_2 = 2.4 Hz), 7.15 (1H, s), 6.71 (1H, s), 3.97 (3H, s), 3.86 (3H, s), 3.82 (3H, s), 3.78 (3H, s); ^{13}C NMR (125 MHz, DMSO-d_6) δ 176.3, 166.2, 160.9, 159.9, 158.3, 154.7, 152.3, 140.6, 140.5, 136.7, 132.0, 130.3, 130.2, 124.1, 122.1, 120.6, 118.4, 118.2, 113.7, 112.9, 108.4, 97.8, 62.6, 61.7, 57.2, 56.1; HRMS: *m/z* calculated for $C_{26}H_{24}NO_7$: 462.1553 $[M+H]^+$. Found 462.1516.

4-Methoxy-*N*-(3-(5,6,7-trimethoxy-4-oxo-4*H*-chromen-2-yl)phenyl)benzamide (4j)

Yield 83%; solid; mp: 179.7 °C;^{1}H NMR (400 MHz, DMSO-d_6) δ 10.30 (1H, s), 8.39 (1H, s), 8.01 (3H, d, J = 8.6 Hz), 7.78 (1H, d, J = 7.8 Hz), 7.54 (1H, t, J = 8.0 Hz), 7.15 (1H, s), 7.10 (2H, d, J = 8.7 Hz), 6.70 (1H, s), 3.97 (3H, s), 3.86 (3H, s), 3.82 (3H, s), 3.78 (3H, s); ^{13}C NMR (125 MHz, DMSO-d_6) δ 176.3, 165.8, 162.8, 161.0, 158.4, 154.7, 152.3, 140.7, 140.6, 132.0, 130.4,

130.1, 127.2, 124.0, 121.9, 118.4, 114.4, 112.9, 108.4, 97.9, 62.6, 61.7, 57.2, 56.2; HRMS: *m/z* calculated for $C_{26}H_{24}NO_7$: 462.1553 $[M+H]^+$. Found 462.1586.

***N*-(3-(5,6,7-Trimethoxy-4-oxo-4*H*-chromen-2-yl)phenyl)benzo[*d*][1,3]dioxole-5-carboxamide (4k)**

Yield 91%; solid; mp: 123.0 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.27 (1H, s), 8.37 (1H, s), 8.00 (1H, d, J = 8.1 Hz), 7.78 (1H, d, J = 7.8 Hz), 7.63 (1H, d, J = 8.2 Hz), 7.56 (1H, s), 7.54 (1H, t, J = 8.0 Hz), 7.15 (1H, s), 7.09 (1H, d, J = 8.0 Hz), 6.70 (1H, s), 6.15 (2H, s), 3.97 (3H, s), 3.82 (3H, s), 3.78 (3H, s); ^{13}C NMR (125 MHz, DMSO-d_6) δ 176.3, 165.4, 160.9, 158.3, 154.7, 152.3, 151.0, 148.1, 140.6, 132.0, 130.1, 129.0, 123.9, 123.7, 121.9, 118.3, 112.9, 108.7, 108.4, 102.6, 97.8, 62.6, 61.7, 57.2; HRMS: *m/z* calculated for $C_{26}H_{22}NO_8$: 476.1340 $[M+H]^+$. Found 476.1311.

4.1.9. General procedure for preparation of 3-(5-hydroxy-6,7-dimethoxy-4-oxo-4*H*-chromen-2-yl)-*N*-substitutedbenzamides (4l–u)

Boron trichloride (1 M solution in dichloromethane, 0.57 mL, 0.57 mmol, 3 equivalents) was slowly added to a cooled solution at 0 °C of the appropriate compound (**4a–k**) (0.19 mmol) in dichloromethane (3 mL). After stirring for 30 min at 0 °C, it was further stirred for 3 h at room temperature. After completion, the reaction was quenched with methanol. The mixture was concentrated and purified by silica gel column chromatography. The compound was further purified by recrystallization from dichloromethane/*n*-hexane to give the titled compounds (**4l–u**).

3-**(5-Hydroxy-6,7-dimethoxy-4-oxo-4*H*-chromen-2-yl)-*N*-phenylbenzamide (4l)**

Yield 90%; solid; mp: 236.2 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.73 (1H, s), 10.42 (1H, s), 8.61 (1H, br s), 8.30 (1H, d, J = 7.4 Hz), 8.17 (1H, d, J = 7.4 Hz), 7.80 (2H, d, J = 7.4 Hz), 7.75

(1H, t, *J* = 7.4 Hz), 7.40 (2H, t, *J* = 7.4 Hz), 7.18–7.13 (2H, m), 7.01 (1H, s), 3.95 (3H, s), 3.76 (3H, s); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 182.3, 164.5, 162.7, 158.9, 152.8, 152.0, 138.9, 135.8, 132.0, 131.2, 130.8, 129.4, 129.2, 128.7 (2C), 125.3, 123.9, 120.5 (2C), 105.6, 105.4, 91.8, 60.0, 56.5; HRMS: *m/z* calculated for $C_{24}H_{20}NO_6$: 418.1285 $[M+H]^+$. Found 418.1314.

3-(5-Hydroxy-6,7-dimethoxy-4-oxo-4*H*-chromen-2-yl)-*N*-(2-methoxyphenyl)benzamide (4m)

Yield 75%; solid; mp: 196.0 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.74 (1H, s), 9.77 (1H, s), 8.65 (1H, br s), 8.30 (1H, d, *J* = 7.7 Hz), 8.16 (1H, d, *J* = 7.7 Hz), 7.75–7.70 (2H, m), 7.24 (1H, t, *J* = 8.0 Hz), 7.20 (1H, s), 7.13 (1H, d, *J* = 8.0 Hz), 7.02–7.00 (2H, m), 3.95 (3H, s), 3.85 (3H, s), 3.76 (3H, s); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 183.1, 164.9, 163.5, 159.7, 153.5, 152.8, 152.7, 136.0, 132.7, 131.8, 131.6, 130.2, 129.9, 127.1, 127.0, 126.1, 126.0, 120.9, 112.2, 106.3, 106.1, 92.5, 60.8, 57.2, 56.4; HRMS: *m/z* calculated for $C_{25}H_{22}NO_7$: 448.1396 $[M+H]^+$. Found 448.1407.

3-(5-Hydroxy-6,7-dimethoxy-4-oxo-4*H*-chromen-2-yl)-*N*-(3-methoxyphenyl)benzamide (4n)

Yield 71%; solid; mp: 117.4 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.74 (1H, s), 10.40 (1H, s), 8.60 (1H, br s), 8.31 (1H, d, *J* = 7.8 Hz), 8.16 (1H, d, *J* = 7.8 Hz), 7.75 (1H, t, *J* = 7.8 Hz), 7.50 (1H, br s), 7.39 (1H, d, *J* = 8.1 Hz), 7.29 (1H, t, *J* = 8.1 Hz), 7.19 (1H, s), 7.02 (1H, s), 6.73 (1H, dd, *J* = 8.1, 2.0 Hz), 3.95 (3H, s), 3.78 (3H, s), 3.76 (3H, s); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 182.3, 164.6, 162.7, 159.4, 158.9, 152.8, 152.0, 140.0, 135.7, 132.0, 131.1, 130.8, 129.5, 129.4, 129.2, 125.3, 112.7, 109.4, 106.2, 105.6, 105.4, 91.8, 60.0, 56.5, 55.0; HRMS: *m/z* calculated for $C_{25}H_{22}NO_7$: 448.1396 $[M+H]^+$. Found 448.1402.

3-(5-Hydroxy-6,7-dimethoxy-4-oxo-4*H*-chromen-2-yl)-*N*-(4-methoxyphenyl)benzamide (4o)

Yield 75%; solid; mp: 228.3 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.74 (1H, s), 10.31 (1H, s), 8.60 (1H, br s), 8.29 (1H, d, J = 7.0 Hz), 8.16 (1H, d, J = 7.0 Hz), 7.73–7.69 (3H, m), 7.18 (1H, s), 7.01 (1H, s), 6.97 (2H, d, J = 8.0 Hz), 3.95 (3H, s), 3.76 (6H, s); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 183.1, 164.8, 163.5, 159.7, 156.5, 153.5, 152.7, 136.5, 132.8, 132.6, 131.8, 131.5, 130.1, 129.8, 125.9, 122.9, 114.5, 106.3, 106.1, 92.5, 60.7, 57.2, 55.9; HRMS: *m/z* calculated for $C_{25}H_{22}NO_7$: 448.1396 $[M+H]^+$. Found 448.1463.

3-(5-Hydroxy-6,7-dimethoxy-4-oxo-4*H*-chromen-2-yl)-*N*-(3,4-methylenedioxyphenyl)benzamide (4p)

Yield 36%; solid; mp: 240.0 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.73 (1H, s), 10.37 (1H, s), 8.57 (1H, br s), 8.29 (1H, d, J = 7.8 Hz), 8.13 (1H, d, J = 7.8 Hz), 7.74 (1H, t, J = 7.8 Hz), 7.44 (1H, d, J = 1.7 Hz), 7.20 (1H, dd, J = 8.4, 1.7 Hz), 7.15 (1H, s), 7.01 (1H, s), 6.94 (1H, d, J = 8.4 Hz), 6.03 (2H, s), 3.95 (3H, s), 3.76 (3H, s); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 183.0, 165.1, 163.5, 159.7, 153.5, 152.6, 147.7, 144.2, 136.3, 133.7, 132.7, 131.8, 131.5, 130.2, 129.9, 125.9, 114.5, 108.7, 106.2, 106.1, 103.4, 101.8, 92.5, 60.8, 57.2.

3-(5-Hydroxy-6,7-dimethoxy-4-oxo-4*H*-chromen-2-yl)-*N*-(3,4,5-trimethoxyphenyl)benzamide (4q)

Yield 26%; solid; mp: 261.2 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 12.56 (1H, s), 8.38 (1H, s), 8.24 (1H, br s), 8.03 (1H, d, J = 7.8 Hz), 7.95 (1H, d, J = 7.8 Hz), 7.64 (1H, t, J = 7.8 Hz), 7.08 (2H, s), 6.62 (1H, s), 6.55 (1H, s), 3.95 (3H, s), 3.90 (9H, s), 3.86 (3H, s); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 182.5, 164.8, 162.7, 159.2, 153.5 (2C), 153.2, 152.8, 136.2, 135.1, 133.9, 132.8, 131.9, 130.1, 129.6, 129.1, 125.0, 106.2, 106.1, 98.0, 90.9, 61.0, 60.9, 56.5, 56.2 (2C); HRMS: *m/z* calculated for $C_{27}H_{26}NO_9$: 508.1602 $[M+H]^+$. Found 508.1614.

2-Methoxy-*N*-(3-(5,6,7-trimethoxy-4-oxo-4*H*-chromen-2-yl)phenyl)benzamide (4r)

Yield 46%; solid; mp: 196.3 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.76 (1H, s), 10.34 (1H, s), 8.40 (1H, s), 7.97 (1H, d, J = 7.9 Hz), 7.80 (1H, d, J = 7.7 Hz), 7.68 (1H, d, J = 7.0 Hz), 7.58–7.49 (2H, m), 7.20 (1H, d, J = 8.4 Hz), 7.08 (1H, t, J = 7.4 Hz), 6.91 (1H, s), 6.85 (1H, s), 3.93 (6H, s), 3.74 (3H, s); ^{13}C NMR (125 MHz, DMSO-d_6) δ 182.9, 165.5, 164.1, 159.6, 157.3, 153.5, 152.8, 140.5, 133.0, 132.7, 131.8, 130.5, 130.3, 125.2, 124.0, 122.4, 121.3, 118.0, 112.7, 106.1, 105.9, 92.2, 60.7, 57.2, 56.7; HRMS: *m/z* calculated for $C_{25}H_{22}NO_7$: 448.1396 $[M+H]^+$. Found 448.1433.

3-Methoxy-*N*-(3-(5,6,7-trimethoxy-4-oxo-4*H*-chromen-2-yl)phenyl)benzamide (4s)

Yield 89%; solid; mp: 197.2 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.75 (1H, s), 10.43 (1H, s), 8.42 (1H, s), 8.02 (1H, d, J = 8.0 Hz), 7.81 (1H, d, J = 7.8 Hz), 7.61–7.51 (3H, m), 7.47 (1H, t, J = 8.0 Hz), 7.18 (1H, dd, J_1 = 8.1 Hz, J_1 = 1.4 Hz), 6.90 (1H, s), 6.85 (1H, s), 3.93 (3H, s), 3.85 (3H, s), 3.74 (3H, s); ^{13}C NMR (125 MHz, DMSO-d_6) δ 182.9, 166.1, 164.1, 159.9, 159.6, 153.5, 152.8, 140.5, 136.6, 132.7, 131.7, 130.3, 130.2, 124.6, 122.5, 120.6, 118.7, 118.2, 113.7, 106.1, 105.8, 92.1, 60.7, 57.2, 56.1; HRMS: *m/z* calculated for $C_{25}H_{22}NO_7$: 448.1396 $[M+H]^+$. Found 448.1432.

4-Methoxy-*N*-(3-(5,6,7-trimethoxy-4-oxo-4*H*-chromen-2-yl)phenyl)benzamide (4t)

Yield 78%; solid; mp: 217.8 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.75 (1H, s), 10.28 (1H, s), 8.41 (1H, s), 8.04–7.96 (3H, m), 7.76 (1H, d, J = 7.5 Hz), 7.51 (1H, t, J = 7.8 Hz), 7.07 (2H, J = 8.2 Hz), 6.86 (1H, s), 6.82 (1H, s), 3.92 (3H, s), 3.84 (3H, s), 3.74 (3H, s); ^{13}C NMR (125 MHz, DMSO-d_6) δ 182.9, 165.8, 164.2, 162.8, 159.5, 153.4, 152.8, 140.8, 132.7, 131.6, 130.4, 130.1,

127.2, 124.4, 122.2, 118.6, 114.3, 106.1, 105.7, 92.1, 60.7, 57.1, 56.4; HRMS: *m/z* calculated for $C_{25}H_{22}NO_7$: 448.1396 $[M+H]^+$. Found 448.1426.

***N*-(3-(5,6,7-Trimethoxy-4-oxo-4*H*-chromen-2-yl)phenyl)benzo[*d*][1,3]dioxole-5-carboxamide (4u)**

Yield 90%; solid; mp: 223.0 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.74 (1H, s), 10.26 (1H, s), 8.39 (1H, s), 8.00 (1H, d, *J* = 7.6 Hz), 7.76 (1H, d, *J* = 7.4 Hz), 7.61 (1H, d, *J* = 7.9 Hz), 7.54 (1H, s), 7.50 (1H, t, *J* = 7.7 Hz), 7.06 (1H, d, *J* =8.0 Hz), 6.85 (1H, s), 6.81 (1H, s), 6.13 (2H, s), 3.92 (3H, s), 3.73 (3H, s); ^{13}C NMR (125 MHz, DMSO-d_6) δ 182.9, 165.3, 164.1, 159.5, 153.4, 152.8, 150.9, 148.1, 140.7, 132.7, 131.6, 130.1, 128.9, 124.4, 123.7, 122.3, 118.6, 108.6, 108.4, 106.0, 105.7, 102.6, 92.1, 60.7, 57.1.

4.2.Biological evaluations

4.2.1. Cytotoxicity evaluation against human cancer cell lines

Screening against the cancer cell lines was carried out at the National Cancer Institute (NCI), Bethesda, Maryland, USA, according to the known standard NCI protocol as described in the supplementary data [59, 60].

4.2.2. Cytotoxicity evaluation against normal human cell lines

Evaluation of cytotoxicity against normal human cell lines was carried according to the well-known method of MTT as described in the supplementary data.

4.2.3. Mechanistic studies

The employed materials and protocols for the conducted mechanistic studies were according to the well-known protocols as described in the supplementary data.

Acknowledgement:

This work was supported by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (#2012-006431). The authors acknowledge the developmental therapeutic program at national cancer institute for performing NCI-60 human tumor cell lines screen of our compounds.

Declaration of interest

The authors report no declarations of interest.

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Highlights

- Design, synthesis and *in vitro* antiproliferative evaluation of 3,5,4'-trimethoxystilbene-5,6,7-trimethoxyflavone hybrids.
- Compounds **4f**, **4h**, **4k** and **4q** elicited promising broad spectrum antiproliferative activity.
- Compounds **4f**, **4h**, **4k** and **4q** were more selective to cancer cells rather than normal cells.
- Compound **4f** triggered cell death via induction of apoptosis in HeLa cells
- Compounds **4f**, **4h**, **4k** and **4q** might be potential leads for development of natural-products based anticancer agents.