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Antiproliferative Activity, Cell-Cycle Dysregulation, and Cellular Differentiation: Salicyl- and Catechol-Derived Acyclic 5-Fluorouracil *O,N*-Acetals against Breast Cancer Cells

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*Herein we report the preparation and biological activity of three compounds with the general formula 1-[2-(5-substituted-2-hydroxybenzyloxy)-1-methoxyethyl]-5-fluorouracil. A catechol-derived compound such as 1-[3-(2-hydroxyphenoxy)-1-methoxypropyl]-5-fluorouracil and two salicyl-derived compounds such as (Z)-1-[4-(2-hydroxyphenyl)-1-methoxybut-3-enyl]-5-fluorouracil [(Z)-**11**] and its dihydrogenated derivative 1-[4-(2-hydroxyphenyl)-1-methoxybutyl]-5-fluorouracil were prepared to complete the set of six* O,N*-acetals. The most active compound against the MCF-7 breast cancer cell line was (Z)-**11**: $IC_{50} = 9.40 \pm 0.64\ \mu M$. Differentiated breast cancer cells generate fat deposits in the cytoplasm. MCF-7 cells treated with (Z)-**11** underwent an increase in lipid content relative to control cells after three days of treatment. Our results suggest that there may be significant potential advantages in the use of this new differentiating agent for the treatment of breast cancer.*

Introduction

5-Fluorouracil (5-FU (**1**), Figure 1) is an example of a rationally designed anticancer agent. The observation that rat hepatomas use radiolabeled uracil more avidly than nonmalignant tissues implies that the enzymatic pathways for the use of uracil differ between malignant and normal cells.[1] In this molecule, the hydrogen atom in position 5 of uracil is replaced by the similar-sized atom of fluorine, and is designed to occupy the active sites of enzymes, thereby blocking metabolism in malignant cells. Although this antimetabolite is toxic, its efficacy makes it one of the most widely used agents against solid tumors.

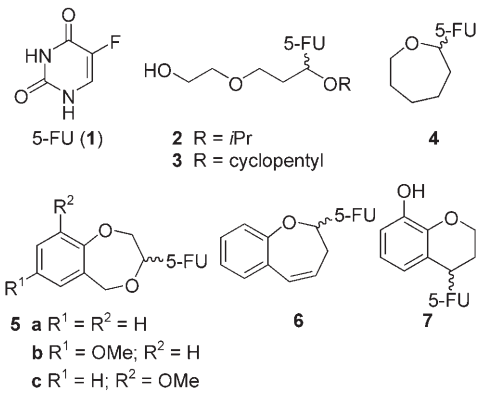

Figure 1. Structures of 5-FU and of 5-FU derivatives previously reported by us. In all cases, the attachment of the 5-FU moiety occurs at the N1 atom of the uracil ring.

An attractive approach to cancer treatment is differentiation therapy, which assumes that neoplastic transformation reveals the inability of a cell population to couple proliferation and differentiation signals. Induced differentiation modulates the cell program by transforming malignant cells into mature cells with no proliferative potential. Therefore, the addition of differentiation treatment to chemotherapy greatly improves the chance of long-term survival.[2] Novel derivatives of 5-FU that possess a broader spectrum of antitumor activity and fewer side effects than 5-FU have been diligently sought by a number of research groups. Campos et al.[3] synthesized a novel class of 5-FU-containing derivatives. The antitumor activi-

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ty of these 5-FU *O*,*N*-acetals was assessed against HEp human cells, showing that compound **2** is fourfold more active than 5-FU. Moreover, **3** has lower toxicity than 5-FU and is able to induce myogenic differentiation in rhabdomyosarcoma cells, suggesting that this drug might be useful for differentiation therapy in this type of tumor (Figure 1). Afterwards, we embarked on a program to synthesize a wide range of 5-FU derivatives linked to saturated annelated seven-membered rings.[4,5] Nevertheless, because the systematic study of structure–activity relationships in seven-membered 5-FU *O*,*N*-acetals has not yet been realized, it is thought that a great effort should be made to search for new anticancer agents in this class of derivatives. Compound **4** has been previously described by us.[6,3] With the idea of increasing the lipophilicity of **4**, we proposed a series of bioisosteric benzannelated seven-membered *O*,*N*-acetals such as **5a**–**c** and **6**[7] (Figure 1). Moreover, the preparation of a benzannelated six-membered derivative such as **7**[7] could emphasize the significance of the biological activity of the seven-membered 5-FU *O*,*N*-acetals. In all cases the attachment of the 5-FU moiety occurs at the N1 position of the pyrimidine ring.

We recently reported the synthesis and anticancer properties, among others, of 1-[2-(5-substituted-2-hydroxymethylphenoxy)-1-methoxyethyl]-5-fluorouracils (compounds **8**, Figure 2).[8] These molecules are characterized by the presence of ether-linked phenoxy and hydroxymethyl groups in an *ortho* relationship. The cytotoxic activity of molecules **8a**–**e** was assessed against the MCF-7 human breast cancer cell line. Herein we report the preparation and biological activity of regioisomers of **8**, that is, compounds characterized by the presence of a free phenolic group and an ether-linked methyloxy moiety, with the general formula 1-[2-(5-substituted-2-hydroxybenzyloxy)-1-methoxyethyl]-5-fluorouracils (compounds **9a**,**d**,**e**, Figure 2), 1-[3-(2-hydroxyphenoxy)-1-methoxypropyl]-5-fluorouracil (**10**), (*Z*)-1-[4-(2-hydroxyphenyl)-1-methoxybut-3-enyl]-5-fluorouracil (**11**), and its dihydrogenated derivative 1-[4-(2-hydroxyphenyl)-1-methoxybutyl]-5-fluorouracil (**12**).

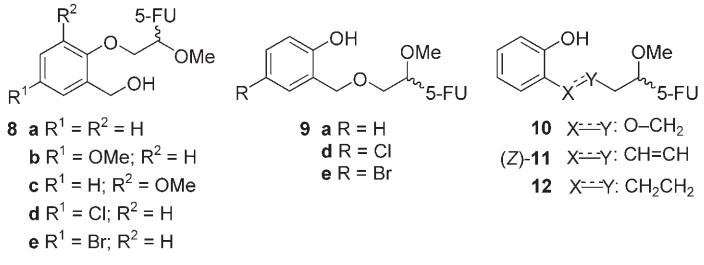
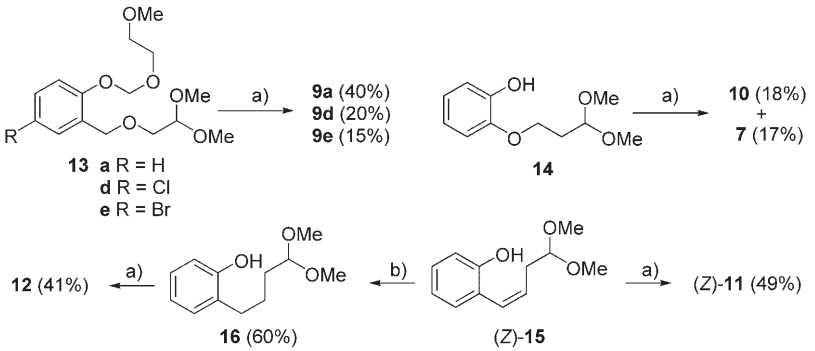


Figure 2. Structures of the acyclic 5-FU *O*,*N*-acetals previously reported by us (**8a**–**e**), and of the 5-FU derivatives reported herein [**9a**,**d**,**e**, **10**, (*Z*)-**11**, and **12**]. In all cases, the attachment of the 5-FU moiety occurs at the N1 atom of the uracil ring.

Chemistry

The synthesis of derivatives **9a**,**d**,**e** was undertaken by previous 2-methoxyethoxymethyl (MEM) protection of the phenolic OH group in the precursor to produce compounds **13a**,**d**,**e**.[9] The unprotected hydroxy group was then alkylated with bromoacetaldehyde dimethyl acetal,[9] and substitution of the OMe group by the 5-FU moiety with concomitant deprotection of the phenolic OH group was accomplished in a single-step, one-pot reaction (Scheme 1).

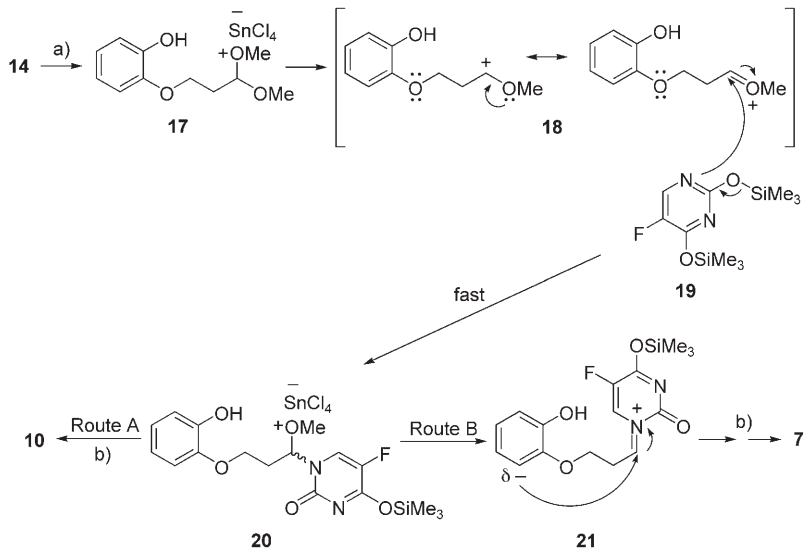
Substitution of the acetal methoxy group with 5-FU was carried out in the presence of the silylating agents 1,1,1,3,3,3-hexamethyldisilazane (HMDS) and trimethylchlorosilane (TCS) under acid catalysis ($SnCl_4$) in dry acetonitrile for 24 h. All condensations were carried out in a one-pot reaction under our standard conditions.[7] A catechol-derived compound such as 1-[3-(2-hydroxyphenoxy)-1-methoxypropyl]-5-fluorouracil (**10**) and two salicyl-derived compounds such as (*Z*)-1-[4-(2-hydroxyphenyl)-1-methoxybut-3-enyl]-5-fluorouracil [(*Z*)-**11**] and its dihydrogenated derivative 1-[4-(2-hydroxyphenyl)-1-methoxybutyl]-5-fluorouracil (**12**) were prepared to complete the set of six acyclic *O*,*N*-acetals (Scheme 1).



Scheme 1. Reagents and conditions: a) 5-FU, HMDS, TCS, $SnCl_4/CH_2Cl_2$, MeCN; b) H_2, Pd/C, 440 kPa, 30 min.

Notably, the condensation reaction between **14** and 5-FU led to the expected acyclic *O*,*N*-acetal **10** as well as the unexpected six-membered 5-FU derivative **7**, in a 1:1 ratio. We previously reported the regiospecific formation of **7** (44%) when starting from 4-methoxychroman-8-ol.[7] A possible explanation for the formation of **10** and **7** (Scheme 2) is the following: The Lewis acid $SnCl_4$ favors the formation of intermediate **18** after complexation on one of the acetal OMe groups. A nucleophilic attack from the bis(trimethylsilyl)-5-fluorouracil derivative **19** then takes place on **18** to produce **20** in a rapid step. From here, two processes compete and two alternative routes may be followed:

A) The aqueous workup gives rise to the acyclic *O*,*N*-acetal **10**.
B) Intermediate **20** may suffer an intramolecular cyclization through the iminium ion intermediate



Scheme 2. Reaction mechanism between **14** and 5-FU; reagents and conditions: a) 5-FU, HMDS, TCS, $SnCl_4/CH_2Cl_2$, MeCN; b) H_2O.

21. In this process, the electron density of the unsubstituted aromatic carbon atom adjacent to the ether oxygen atom is determinant and favored by the ether oxygen atom of the alkyl chain. Finally, after hydrolysis, **7** is formed. A similar iminium ion was hypothesized by Hager and Liotta[10] during the formation of the β anomer of AZT from a non-carbohydrate precursor.

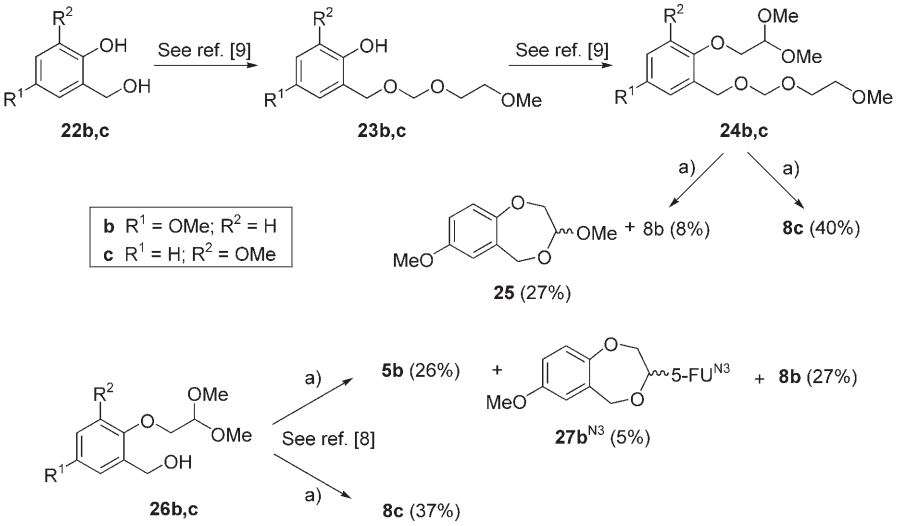
Compound (*Z*)-**15** was reported by us.[7] Its catalytic hydrogenation under 10% Pd/C gave rise to **16**. The corresponding condensation reactions produced (*Z*)-**11** (49%) and **12** (41%), respectively (Scheme 1).

Notably, compound **8b** was obtained with the highest yield (26%)[8] with **26b** as the starting *O*,*O*-acetal and not the MEM-containing *O*,*O*-acetal **24b** (8%). Nevertheless, **8c** was obtained in approximately the same yield regardless of the nature of the starting *O*,*O*-acetals: 40% from **24c** and 37% from **26c**[8] (Scheme 3).

Results and Discussion

The anticancer activity of 5-FU, 5-FU acyclic *O*,*N*-acetals **9a**,**d**,**e**, **10**, (*Z*)-**11**, and **12** are shown in Table 1 (entries 2, 6–11). Also shown are the biological activities of **8a**, **8d**, and **8e**[8] (entries 3–5), in order to establish the primary structure–activity studies related to the regioisomerism of the compounds.

We performed a parallel study of IC_{50}, cell-cycle effects, and apoptosis that result from treatment with 5-FU alone (Table 1, entry 2). 5-FU has an IC_{50} value of $2.75 \pm 0.28\,\mu M$ against the MCF-7 cell line, and when the cancerous cells were treated with a concentration equal to the IC_{50} value of 5-FU, they gathered in the S phase, and a significant induction of apoptosis was observed. Structure **8a** (Table 1, entry 2) is ~2.4-fold more active than its regioisomer **9a** (Table 1, entry 6). Nevertheless, each pair of the regioisomers **8d** (Table 1, entry 4) and **9d** (Table 1, entry 7), and **8e** (Table 1, entry 5) and **9e** (Table 1, entry 8) are equipotent. Taking **8a** as a lead, the strategy of the functional CH_2–O inversion (O–CH_2) was applied, and consequently the antiproliferative activity of **10** was measured: **10** (Table 1, entry 9) is slightly less active than **8a** (Table 1, entry 3). Next, exchange of the ether O atom and the methylene group was undertaken to produce **12**. From Table 1, entry 11, it appears that the absence of the ether oxygen atom in the side chain is not detrimental to the antiproliferative activity (compare the data in entries 6 and 11). To improve the antiproliferative activity of **12**, compound (*Z*)-**11** was designed and synthesized with the objective to produce an analogue in which the side chain is conformationally restricted around the C3′–C4′ bond by the presence of a double bond with the *Z* configuration. An increase in the antiproliferative activity of (*Z*)-**11** was observed ($IC_{50} = 9.40 \pm$



Scheme 3. Reagents and conditions: a) 5-FU, HMDS, TCS, $SnCl_4/CH_2Cl_2$, MeCN. The identification tag $\mathbf{27b}^{N3}$ indicates that the 5-FU moiety is linked to the carbon chain through its N3 atom.

Table 1. Antiproliferative activities, cell-cycle dysregulation, and apoptosis induction in the MCF-7 human breast cancer cell line after treatment with the compounds for 24 or 12 and 48 h.

Entry	Compd	IC_{50} [μM][a]	Cell Cycle (48 h)[b]			Apoptosis [%][c]	
			G_0/G_1	S	G_2/M	24 or 12 h	48 h
1	Control		68.39	12.04	19.57	1.07 ± 0.22	1.23 ± 0.13
2	5-FU	2.75 ± 0.28	58.07	39.83	2.10	66.33 ± 2.76[e]	92.56 ± 3.57
3	**8a**[d]	18.5 ± 0.95	67.18	4.67	28.16	59.90 ± 2.65[f]	40.23 ± 1.98
4	**8d**[d]	18.0 ± 0.85	71.01	28.99	0.00	44.36 ± 2.01[f]	50.64 ± 2.24
5	**8e**[d]	16.0 ± 1.18	51.45	20.66	27.88	42.24 ± 1.89[f]	36.37 ± 1.68
6	**9a**	44.0 ± 1.26	64.51	25.39	10.10	3.60 ± 0.25[e]	2.06 ± 0.11
7	**9d**	18.0 ± 0.92	72.15	20.52	7.33	7.15 ± 0.97[e]	1.15 ± 0.12
8	**9e**	18.0 ± 1.05	72.45	18.17	9.38	2.71 ± 0.37[e]	1.86 ± 0.20
9	**10**	27.0 ± 1.13	63.71	29.67	6.62	3.17 ± 0.13[e]	1.68 ± 0.35
10	(*Z*)-**11**	9.40 ± 0.64	55.76	42.33	1.91	1.36 ± 0.25[e]	1.70 ± 0.15
11	**12**	42.0 ± 2.33	66.29	33.71	0.00	2.18 ± 0.27[e]	1.56 ± 0.53

[a] Data determined according to Ref. [24]. [b] Data determined by flow cytometry.[25] [c] Apoptosis was determined using an annexin-V-based assay.[25] The data indicate the percentage of cells undergoing apoptosis in each sample. All experiments were conducted in duplicate and gave similar results. The data are the mean values ± SEM of three independent determinations. [d] Data (antiproliferative activity, cell-cycle distribution, and apoptosis) taken from Ref. [7]. [e] 12 h. [f] 24 h.

It is well established that different anticancer drugs may exert their therapeutic effects by combining differentiating and cytotoxic actions in several types of cancer.[12,13] Genes that are altered in neoplasia affect three important biological routes that normally regulate cell growth and tissue homeostasis: cell cycle, apoptosis, and differentiation. Although each of these routes can be defined by means of a unique set of molecular events, they are intimately interrelated in such a way that if one of them is disturbed, profound consequences can be produced in the others.[14] The new compounds may therefore affect any of these routes in a more specific way, giving rise to the antitumor effect.

0.64 μM, entry 10). In comparing structures **12** and (*Z*)-**11**, it is worth emphasizing that the introduction of the double bond in compound (*Z*)-**11** increased the antiproliferative activity ~4.5-fold ($IC_{50} = 9.40 \pm 0.64$ μM, entry 10 versus $IC_{50} = 42.0 \pm 2.33$ μM, entry 11).

Correlation between antiproliferative activity of compounds **9a,d,c**, **10** and (*Z*)-**11** and their calculated lipophilicities by the CDR option of the PALLAS 2.0 program[11] (Table 2) was obtained [Eq. (1)]:

$$p(IC_{50}) = 4.20(\pm 0.04) + 0.46(\pm 0.04)\ \mathrm{clog}\,P$$
$$n = 5,\ r^2 = 0.976,\ s = 0.044,\ F_{1,3} = 124.26,\ \alpha < 0.001 \qquad (1)$$

outlier not included: **12**

for which $p(IC_{50}) = -\log(IC_{50})$ (bearing in mind that a higher $p(IC_{50})$ value means a more potent compound), n is the number of compounds, r^2 is the correlation coefficient, s is the standard deviation, F is the ratio between the variances of observed and calculated activities, and data within parentheses are standard errors of estimate. In the derivation of Equation (1) the more lipophilic compound **12** (Table 2, $\mathrm{clog}\,P = 1.84$) was not included, as it was found to be a misfit in the correlation. It could be hypothesized that the low antiproliferative activity of **12** is attributed to the high flexibility of its side chain orientation.

Table 2. Antiproliferative activities in the MCF-7 human breast cancer cell line after treatment, and calculated lipophilicity values of the compounds.

	9a	**9d**	**9e**	**10**	(*Z*)-**11**	**12**
$p(IC_{50})$[a]	4.36	4.74	4.74	4.57	5.03	4.38
clog *P*[b]	0.32	1.06	1.26	0.87	1.76	1.84

[a] $p(IC_{50}) = -\log(IC_{50})$; higher values of $p(IC_{50})$ reflect greater compound potency. [b] Calculated by the CDR option of the PALLAS 2.0 program.[11]

Once the antiproliferative activity was determined, we decided to check the modifications that the various compounds provoke in the cell cycle. This study showed differences in the pattern of behavior depending on the structural changes carried out. Whereas **8a** concentrated the cancerous cells in the G_2/M phase (Table 1, entry 3), **8d** gathered the cells in the S phase (Table 1, entry 4). Meanwhile, **8e** distributed the cancerous cells between the S and G_2/M phases (Table 1, entry 5). Nevertheless, compounds **9a**, **10**, (*Z*)-**11**, and **12** concentrated the cells in the S phase, whilst **9d** and **9e** gathered them in the S and G_0/G_1 phases. With bromine or chlorine (compounds **9d** and **9e**, respectively) introduced at position 5 of the benzene ring, the increase in antitumor activity relative to that of **9a** was accompanied by an increase in the percentage of cells that were accumulated in the G_0/G_1 phase of the cell cycle. There was also a significant decrease in the G_2/M phase relative to the control cells. Compound (*Z*)-**11** was the most active (9.4 ± 0.64 μM), giving rise to an arrest of the cells in the S phase of the cell cycle. It promoted a diminution in the percentage of cells in the G_0/G_1 and a decrease to the total disappearance of cells in the G_2/M phase.

We then carried out apoptosis assays using an annexin-V-based assay and flow cytometry at various treatment times. In response to **8a**, **8d**, and **8e** (Table 1, entries 3–5), the percentage of apoptotic cells increased from 1.07% in control cells to a maximum of 59.90% (**8a**), 44.36% (**8d**), and 42.24% (**8e**) apoptotic cells (24 h) at a concentration equal to their IC_{50} values against the MCF-7 cell line. Nevertheless, compounds **9a**, **9d**, **9e**, **10**, (*Z*)-**11**, and **12** gave rise to only very slight increases in the apoptotic cells, as can be seen from Table 1. Such augmentations were more evident in the first 12 h and for compounds **9a** and **9d**. The modifications were minimal after 48 h, with **9d** being the structure with which more apoptotic cells appeared (Table 1, entry 7). From the data shown in

Table 1, it can be concluded that compounds **9a**, **9d**, **9e**, **10**, (*Z*)-**11**, and **12** do not act preferentially through an apoptotic mechanism in human breast cancer cells.

Finally, our aim was to identify the compounds capable of inducing cell differentiation on MCF-7 human breast cancer cells. Differentiated breast cancer cells display properties that are associated with lactation and include the generation of fat deposits within the cytoplasm.[15] The percentage of lipid-positive cells was determined by Nile red staining in a flow cytometer. Nile red is a specific fluorescent probe for quantifying the intracellular lipid contents by flow cytometry in mammalian cells.[16]

All the compounds (except **9a**) caused an increase in lipid content over control levels after three days of treatment (Figure 3); however, no differentiating activity was observed by

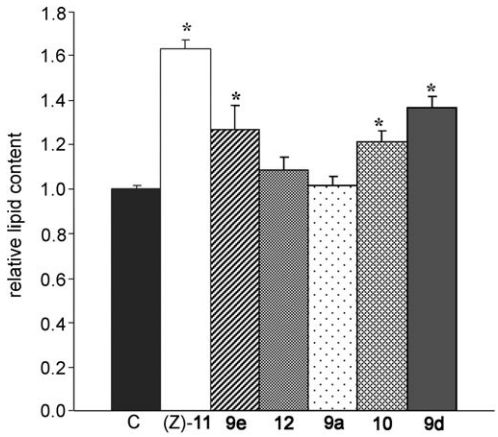


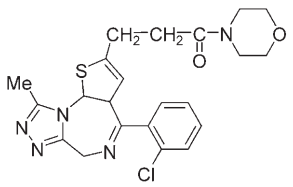
Figure 3. Quantitation of intracellular lipid contents by Nile red staining and flow cytometry after treatment with the compounds indicated at their respective IC_{50} values for three days. Quantities are expressed as the fraction relative to non-treated MCF-7 cells as the control (C). The histogram represents the mean $\pm$SD of three determinations; significant at $^*p < 0.05$ by one-way ANOVA with Dunnett's post-test.

5-FU (Nile red staining assay, data not shown). Compound (*Z*)-**11** increased the percentage of lipids up to values of $>60\%$ those of the untreated cells. Nevertheless, (*Z*)-**11** increased the percentage of cells in the S phase significantly, which might contradict the high level of differentiation. Although G_1 arrest has been the center of attention in differentiation, some reports are concerned with the involvement of G_2/M- and S-phase arrest in this differentiating process.[17,18] Moreover, recent studies have demonstrated that purine nucleotides and nucleosides caused S-phase arrest and differentiation in human cells of chronic myelogenous leukemia.[19] The duplication of the cellular genome during the S phase of the cell cycle is critical because during this process the cells are highly susceptible to the induction of differentiation.[20] Notably, if the 5-FU moiety is linked through its N1 atom to the (*Z*)-1-[4-(2-hydroxyphenyl)-1-methoxybut-3-enyl] fragment to produce (*Z*)-**11**, the resulting compound does not effect an apoptotic response, but an induction of differentiation instead.

Introduction of the bromine and chlorine atoms causes an increase in differentiation levels from 20 to 40% relative to the control. The arrest of cells in the G_0/G_1 phase by these compounds show the passage to a non-proliferative quiescent state that implies entrance into the differentiation process.[21] Others[22] have recently shown that novel quinoline compounds cause lipid droplet accumulation as a phenotypic marker of differentiation, loss of Ki67 antigen expression (a cell-cycle marker indicative of entry into the G_0 phase), and reduced protein levels in the G_1 phase.

These data show that the mechanism by which such compounds exert their antiproliferative effect is the induction of differentiation in the MCF-7 human breast cancer cell line, instead of the induction of apoptosis or cytotoxic action. Compounds caused tumor cells to re-enter the normal pathway of development and thus constitute a new category of experimental breast cancer differentiation agents.

In combination, the anti-estrogen tamoxifen and the vitamin-A-related compound, all-*trans*-retinoic acid, act synergistically to inhibit the growth of MCF-7 human breast cancer cells:[23] in this case, a known anti-neoplastic agent is combined with another structure with differentiating activity. In the case reported herein, the antitumor activities are produced by the capacity of bringing about differentiation. Nevertheless, there are new compounds such as WEB-2086,[24] an antagonist of



WEB-2086

platelet-activating factor receptor (PAFR), with known anti-inflammatory, antiangiogenic, and antileukemic properties that also inhibit proliferation in human solid tumor cell lines of varying histology, and with much higher efficacy than in normal fibroblasts. Both WEB-2086 and (*Z*)-**11** show antitumor properties through differentiating activity.

Conclusions

Although in vivo studies are necessary, the findings of the present study suggest that the new (*Z*)-1-[4-(2-hydroxyphenyl)-1-methoxybut-3-enyl]-5-fluorouracil [(*Z*)-**11**] may be useful as an agent for differentiation therapy against the MCF-7 human breast cancer cell line and might have tumor-selective activity. Our results suggest that there may be significant potential advantages in the use of this new differentiating agent for the treatment of breast cancer. Further studies on increased constraint and modulation of the side chain of (*Z*)-**11** are currently being undertaken to gather important information for structure-based drug design.

Experimental Section

Chemistry

The general methods were the same as those previously described.[7,8]

2-(4,4-Dimethoxybutyl)phenol 16. Compound **15**[7] (5.9 g, 28.4 mmol) was dissolved in MeOH (80 mL) and hydrogenated over 10% Pd/C (500 mg) at 440 kPa for 30 min. The catalyst was filtered, washed (MeOH), and the combined methanolic fractions were concentrated to give a residue which was purified by flash chromatography by using a mixture of diethyl ether/hexanes 3:7. Compound **16** was obtained as a white solid (3.03 g, 60%); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.07 (m, 2H, H_{Ar}), 6.84 (dt, J = 1.2, 7.4 Hz, 1H, H_{Ar}), 6.77 (d, J = 8.0 Hz, 1H, H_{Ar}), 4.80 (s, 1H, OH), 4.46 (t, J = 5.4 Hz, 1H, H-1′), 3.35 (s, 6H, 2×OMe), 2.65 (t, J = 7.2 Hz, 2H, H-4′), 1.69 ppm (m, 4H, H-2′ and H-3′); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 154.14 (C1), 139.3 (C5), 128.1 (C2), 122.23 (C3), 120.43 (C4), 115.52 (C6), 104.97 (C1′), 52.87 (2×OMe), 31.3 (C2′), 29.32 (C4′), 25.13 ppm (C3′); Anal. calcd for $C_{12}H_{18}O_3$: C 68.54, H 8.63, found: C 68.59, H 8.57.

Final compounds

Reaction between the acyclic *O*,*O*-acetals 13a,d,e, 10, (*Z*)-15, 16, 24b, and 24c, and 5-fluorouracil; general procedure. A solution of $SnCl_4/CH_2Cl_2$ (1.0 M, 1.9 mmol) was added dropwise with stirring under argon at room temperature to a suspension of **13a**,**d**,**e**,[9] **14**,[7] (*Z*)-**15**,[7] **16**, **24b**,[8] and **24c**[8] (1.6 mmol), 5-fluorouracil (5-FU, 1.8 mmol), containing trimethylchlorosilane (TCS, 1.6 mmol) and 1,1,1,3,3,3-hexamethyldisilazane (HMDS, 1.6 mmol) in dry acetonitrile (10 mL mmol^{-1}). After stirring for 24 h, the reaction was quenched by the addition of a concentrated aqueous solution of Na_2CO_3. The solution was washed with CH_2Cl_2 (3×20 mL), and the combined organic layers were dried (Na_2SO_4), filtered, and concentrated. The target molecules **9a**,**d**,**e**, **15**, (*Z*)-**11**, **12**, **8b**, and **8c** were purified by flash chromatography by using mixtures of CH_2Cl_2/MeOH 100:1.

(*R*,*S*)-1-[2-(2-Hydroxybenzyloxy)-1-methoxyethyl]-5-fluorouracil 9a. Starting *O*,*O*-acetal: **13a**;[9] yield: 40%; white solid; mp: 154–156 °C; R_f (CH_2Cl_2/MeOH, 10:0.25): 0.23; R_f (CH_2Cl_2/MeOH, 9:0.8): 0.8; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.75 (s, 1H, NH), 7.42 (d, $J_{H,F}$ = 5.7 Hz, 1H, $H_{5\text{-}FU}$), 7.24 (dt, J = 1.7, 7.8 Hz, 1H, H-4 or H-5); 7.10 (dd, J = 1.7, 7.4 Hz, 1H, H-6 or H-3); 7.01 (s, 1H, OH); 6.90 (d, J = 7.8 Hz, 1H, H-3 or H-6); 6.88 (t, J = 7.4 Hz, 1H, H-5 or H-4); 5.78 [dt, J = 1.7, 4.8 Hz, 1H, H-1′ (part X of an ABX system)]; 4.77 (d, J_{gem} = 12.1 Hz, 1H, $PhCH_2$); 4.66 (d, J_{gem} = 12.1 Hz, 1H, $PhCH_2O$); 3.74 [m, 1H, OCH_2CH (part A of an ABX system, J_{AB} = 11 Hz, J_{AX} = 5.1 Hz)]; 3.72 [m, 1H, OCH_2CH (part B of an ABX system, J_{BX} = 4.6 Hz]; 3.42 ppm (s, 3H, OMe); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 157.3 (C2), 155.76 ($C4_{5\text{-}FU}$), 150.03 ($C2_{5\text{-}FU}$), 141.02 (d, J = 239.84 Hz, $C5_{5\text{-}FU}$), 130.22, 129.56 (C6, C4), 123.54 (d, J = 33.65 Hz, $C6_{5\text{-}FU}$), 121.94 (C1), 120.27, 116.66 (C3, C5), 84.96 (C1′), 71.86 ($PhCH_2O$), 69.68 (C2′), 57.40 ppm (OMe); HR LSIMS calcd for $C_{14}H_{15}FN_2O_5Na$: $[M+Na]^+$ 333.0862, found: 333.0862; Anal. calcd for $C_{14}H_{15}FN_2O_5$: C 54.19, H 4.87, N 9.03, found: C 54.35, H 5.23, N 8.86.

(*R*,*S*)-1-[2-(5-Chloro-2-hydroxybenzyloxy)-1-methoxyethyl]-5-fluorouracil 9d. Starting *O*,*O*-acetal: **13d**;[9] yield: 20%; white solid; mp: 106–108 °C; R_f (CH_2Cl_2/MeOH, 9:1): 0.6; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 10.4 (s, 1H, NH), 7.44 (d, $J_{H,F}$ = 5.7 Hz, 1H, $H_{5\text{-}FU}$), 7.11 (s, 1H, OH), 7.10 (dd, J = 2.4, 8.5 Hz, 1H, H-4), 7.07 (d, J = 2.4 Hz, 1H, H-6), 6.79 (d, J = 8.5 Hz, 1H, H-3), 5.74 [t, 1H, H-1′ (part X of an ABX system, J = undetermined)], 4.61 (d, J_{gem} = 12.1 Hz, 1H, $PhCH_2O$), 4.53 (d, J_{gem} = 12.1 Hz, 1H, $PhCH_2O$), 3.72 [m, 1H, OCH_2CH (part A of an ABX system, J_{AB} = 11.1 Hz, J_{AX} = 6.9 Hz)], 3.68 [m, 1H, OCH_2CH (part B of an ABX system, J_{BX} = 4.2 Hz)], 3.39 ppm (s, 3H, OMe); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 157.56 (d, J = 26.2 Hz, $C4_{5\text{-}FU}$), 154.05 ($C2_{5\text{-}FU}$), 150.19 (C2), 140.87 (d, J = 236.9 Hz, $C5_{5\text{-}FU}$), 129.49, 129.21 (C6, C4), 125.70 (C1), 123.45 (d, J = 44 Hz, $C6_{5\text{-}FU}$), 123.73 (C5), 117.60 (C3), 84.98 (C1′), 70.30 (C2′), 69.68 ($PhCH_2O$), 57.28 ppm (OMe); HR LSIMS calcd for $C_{14}H_{14}ClFN_2O_5Na$: $[M+Na]^+$ 367.0472, found: 367.0472; Anal. calcd for $C_{14}H_{14}ClFN_2O_5$: C 48.78, H 4.09, N 8.13, found: C 48.67, H 3.83, N 8.17.

(*R*,*S*)-1-[2-(5-Bromo-2-hydroxybenzyloxy)-1-methoxyethyl]-5-fluorouracil 9e. Starting *O*,*O*-acetal: **13e**;[9] yield: 15%; white solid; mp: 146–148 °C; R_f (CH_2Cl_2/MeOH, 9:0.8): 0.6; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 10.4 (s, 1H, NH), 7.43 (d, $J_{H,F}$ = 5.7 Hz, 1H, $H_{5\text{-}FU}$), 7.32 (dd, J = 2.4, 8.6 Hz, 1H, H-4), 7.22 (d, J = 2.4 Hz, 1H, H-6), 6.79 (d, J = 8.6 Hz, 1H, H-3), 7.08 (s, 1H, OH), 5.77 [dt, 1H, H-1′ (part X of an ABX system, J = 1.3, 4.7 Hz)], 4.71 (d, J_{gem} = 12.2 Hz, 1H, $PhCH_2O$), 4.61 (d, J_{gem} = 12.2 Hz, 1H, $PhCH_2O$), 3.75 [m, 1H, H-2′ (part A of an ABX system, J_{AB} = 11.2 Hz, J_{AX} = 8.52 Hz)], 3.7 [m, 1H, H-2′ (part B of an ABX system, J_{BX} = 6.12 Hz)], 3.44 ppm (s, 3H, OMe); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 156.99 (d, J = 35 Hz, $C4_{5\text{-}FU}$), 154.88 ($C2_{5\text{-}FU}$), 150.00 (C2), 141.07 (d, J = 317 Hz, $C5_{5\text{-}FU}$), 132.80, 131.98 (C6, C4), 124.15 (C1), 123.45 (d, J = 44 Hz, $C6_{5FU}$), 118.52 (C3), 112.07 (C5), 84.94 (C1′), 71.14 (C2′), 69.96 ($PhCH_2O$), 57.50 ppm (OMe); HR EIMS calcd for $C_{14}H_{14}BrFN_2O_5Na$: $[M]^+$ 388.0070, found: 388.0070; Anal. calcd for $C_{14}H_{14}BrFN_2O_5$: C 43.21, H 3.63, N 7.20, found: C 42.98, H 3.67, N 6.87.

(*R*,*S*)-1-[3-(2-Hydroxyphenoxy)-1-methoxypropyl]-5-fluorouracil 10 and (*R*,*S*)-1-(8-hydroxychroman-4-yl)-5-fluorouracil 15. Starting *O*,*O*-acetal: **14**.[7] Two fractions were obtained after purification: **10**, yield: 18%; white solid; mp: 125–127 °C; R_f (CH_2Cl_2/MeOH, 9:0.8): 0.6; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 10.15 (s, 1H, NH), 7.41 (d, $J_{H,F}$ = 5.6 Hz, 1H, $H_{5\text{-}FU}$), 6.86 (m, 4H, H_{Ar}), 6.51 (s, 1H, OH), 5.93 (dt, J = 1.2, 6.5 Hz, 1H, H-1′), 4.21 (ddd, J = 4.4, 6.7, 11.7 Hz, 1H, H-3′a), 4.00 (ddd, J = 4.7, 6.7, 11.7 Hz, 1H, H-3′b), 3.37 (s, 3H, OMe), 2.26 (ddd, J = 6.5, 14.5 Hz, 3rd J value undetermined, 1H, H-2′a), 2.18 ppm (ddd, J = 14.5 Hz, 2nd and 3rd J values undetermined, 1H, H-2′b); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 155.63 (d, J = 26.3 Hz, $C4_{5\text{-}FU}$), 150.06 ($C2_{5\text{-}FU}$), 146.33 (C2), 145.61 (C1), 141.49 (d, J = 238.2 Hz, $C5_{5\text{-}FU}$), 122.80 (d, J = 32.9 Hz, $C6_{5\text{-}FU}$), 122.32 (C5), 120.10 (C4), 115.62 (C6), 113.11 (C3), 85.32 (C1′), 64.56 (C3′), 56.99 (OMe), 34.63 ppm (C2′); HR LSIMS calcd for $C_{14}H_{15}FN_2O_5Na$: $[M+Na]^+$ 333.0862, found: 333.0862; Anal. calcd for $C_{14}H_{15}FN_2O_5$: C 54.19, H 4.87, N 9.03, found: C 53.99, H 4.92, N 8.89. The second fraction was identified as **15**: yield: 17%; its physicochemical properties were identical to those previously reported.[7]

(*R*,*S*)-(*Z*)-1-[4-(2-Hydroxyphenyl)-1-methoxybut-3-enyl]-5-fluorouracil (*Z*)-11. Starting *O*,*O*-acetal: (*Z*)-**15**;[7] yield: 49%; white solid; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 10.09 (s, 1H, NH), 7.42 (d, J = 5.6 Hz, 1H, $H_{5\text{-}FU}$), 7.07 (d, J = 5.1 Hz, 1H, $H_{5\text{-}FU}$), 7.11 (dt, J = 1.6, 7.7 Hz, 1H, H-3 or H-6), 7.03 (d, J = 6.5 Hz, 1H, H-5 or H-4), 6.85 (dd, J = 2.4, 7.7 Hz, 1H, H-6 or H-3), 6.76 (t, J = 6.5 Hz, 1H, H-5 or H-4), 6.6 (d, J = 12.3 Hz, 1H, H-4′), 5.7 (m, 2H, H-1′ and H-4′), 3.31 (s, 3H, OMe), 2.64 ppm (m, 2H, H-2′); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 157.70 (d, J = 26.1 Hz, $C4_{5\text{-}FU}$), 153.28 ($C2_{5\text{-}FU}$), 150.23 (C1), 140.94 (d, J = 237.4 Hz, $C5_{5\text{-}FU}$), 130.25 (d, J = 41.7 Hz, $C6_{5\text{-}FU}$), 129.86 (C4′), 129.04 (C5), 125.00 (C3), 123.05 (C2), 122.88 (C3′), 120.13 (C4), 115.93 (C6), 86.53 (C1′), 57.14 (OMe), 33.67 ppm (C2′); HR LSIMS calcd for $C_{15}H_{15}FN_2O_4Na$: $[M+Na]^+$ 329.0913, found: 329.0913; Anal. calcd for $C_{15}H_{15}FN_2O_4$: C 58.82, H 4.94, N 9.15, found: C 59.01, H 4.65, N 8.85.

(*R*,*S*)-1-[4-(2-Hydroxyphenyl)-1-methoxybutyl]-5-fluorouracil 12. Starting *O*,*O*-acetal: **16**; yield: 41%; white solid; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 10.55$ (s, 1H, NH), 7.33 (d, $J = 5.6$ Hz, 1H, $H_{5\text{-FU}}$), 7.02 (m, 2H, H_{Ar}), 6.79 (m, 2H, H_{Ar}), 6.71 (s, 1H, OH), 5.61 (dt, $J = 1.6$, 5.8 Hz, 1H, H-1′), 3.26 (s, 3H, OMe), 2.65 (m, 2H, H-4′), 1.73 ppm (m, 4H, H-3′ and H-2′); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 157.52$ (d, $J = 34.8$ Hz, $C4_{5\text{-FU}}$), 154.15 ($C2_{5\text{-FU}}$), 150.42 (C1), 141.23 (d, $J = 316.9$ Hz, $C5_{5\text{-FU}}$), 130.25 (C5), 127.53 (C2), 127.32 (C3), 123.30 (d, $J = 43.5$ hz; $C6_{5\text{-FU}}$), 120.32 (C4), 115.51 (C6), 87.4 (C1′), 56.78 (OMe), 33.96 (C4′), 28.81 (C2′), 24.44 ppm (C3′); HR LSIMS calcd for $C_{15}H_{17}FN_2O_4Na$: $[M+Na]^+$ 331.1239, found: 331.1239; Anal. calcd for $C_{15}H_{17}FN_2O_4$: C 58.44, H 5.56, N 9.09, found: C 58.21, H 5.32, N 8.95.

(*R*,*S*)-3,7-Dimethoxy-2,3-dihydro-5*H*-1,4-benzodioxepin 25 and (*R*,*S*)-1-[2-(2-hydroxy-5-methoxybenzyloxy)-1-methoxyethyl]-5-fluorouracil 8b. Starting *O*,*O*-acetal: **24b**.[8] Two fractions were obtained after purification: **25** (27%) and **8b** (8%). Their physicochemical properties were identical to those previously reported: **25** (Ref. [7]) and **8b** (Ref. [8]).

(*R*,*S*)-1-[2-(2-Hydroxy-3-methoxybenzyloxy)-1-methoxyethyl]-5-fluorouracil 8c. Starting *O*,*O*-acetal: **24c**.[8] Compound **8c** (40%) was obtained. Its physicochemical properties were identical to those previously reported.[8]

Biological activity

The results of the antiproliferative activities, apoptosis induction, and cell-cycle distribution are recorded in Table 1.

Cell culture. MCF-7 cells were grown at 37 °C in an atmosphere containing 5% CO_2, with Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with heat-inactivated fetal bovine serum (FBS, 10%, Gibco), L-glutamine (2%), sodium bicarbonate (2.7%), HEPES buffer (1%), gentamicin (40 mg L^{-1}), and ampicillin (500 mg L^{-1}).

Drugs and drug treatments. The drugs were dissolved in DMSO or water and stored at −20 °C. For each experiment, the stock solutions were further diluted in medium to obtain the desired concentrations. The final DMSO solvent concentration in cell culture was $\leq 0.1\%$ *v/v*, a concentration that does not affect cell replication.[25] Parallel cultures of MCF-7 cells in medium with DMSO were used as controls.

Cytotoxicity assays in vitro. The effect of anticancer drugs on cell viability was assessed by using the sulforhodamine-B (SRB) colorimetric assay.[26] Aliquots of MCF-7 cell suspension (30×10^3 cells/well) were seeded onto 24-well plates and incubated for 24 h. The cells were then treated with various concentrations of drugs in the culture medium. Three days later, the wells were aspirated, fresh medium and treatment were added, and cells were maintained for three additional days. Thereafter, cells were processed as described previously,[26] with a Titertek Multiscan apparatus (Flow, Irvine, CA, USA) at $\lambda = 492$ nm. We evaluated the linearity of the SRB assay with cell number for each MCF-7 cell stock before each cell growth experiment. The IC_{50} values were calculated from semi-logarithmic dose–response curves by linear interpolation. All of the experiments were plated in triplicate wells and were carried out at least twice.

Cell-cycle distribution analysis. The cells at 70% confluence were treated with either DMSO alone or with concentrations of the compounds determined by their IC_{50} values. Fluorescence-activated cell sorting (FACS) analysis was performed after 48 h of treatment as described.[25] All experiments were performed in triplicate and yielded similar results.

Apoptosis detection by staining with annexin V–FITC and propidium iodide. The annexin V–FITC apoptosis detection kit I (Pharmingen, San Diego, CA, USA) was used to detect apoptosis by flow cytometry according to Boulaiz et al.[27] All experiments were performed in triplicate and yielded similar results.

Statistical analyses. Data are expressed as mean ± SEM. Comparison between DMSO-treated control cells and drug-treated cells were made by using one-way ANOVA with Dunnett's post-test. Statistical analysis was performed using SPSS version 12.0 software.

Nile red staining and flow cytometric analysis. After treatment with the compounds at their respective IC_{50} values for three days, MCF-7 cells were stained with Nile red dye.[16] The stock solution was made by dissolving Nile red (Sigma–Aldrich) to 1 mg mL^{-1} in DMSO, and the working solution was prepared fresh immediately before use by diluting 100-fold in phosphate-buffered saline (PBS). Prior to staining, the cells were dislodged by trypsinization, washed, and fixed at 40 °C for 15 min in 2% buffered formaldehyde. After fixation, the cells were washed, resuspended in 400 mL PBS, and stained after the addition of 44 μL Nile red working solution on ice for 30 min. For flow cytometry, the cells were washed in PBS, and the analysis was carried out in a Becton–Dickinson Vantage FACS instrument (excitation: $\lambda = 488$ nm, emission: $\lambda = 585$ nm).

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