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Synthesis and Evaluation of New 5-Fluorouracil Antitumor Cell Differentiating Derivatives

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Abstract—Three new antitumour drugs containing two 5-fluorouracil moieties at both ends of the structure and a two amide bond linker were synthesized. Appropriated bis-acetal were reacted with two equivalents of 5-FU to afford the desired compounds. These drugs were evaluated for their ability to induce myogenic maturation in vitro on human rhabdomyosarcoma cells in an experimental model. Compounds **5** and **6** induced morphological and phenotypical differentiation in rhabdomyosarcoma cells at 4.5 and 3.5 μM, respectively. These new cell differentiating agents could be used as an alternative to selective destruction of undifferentiated cells. A potential role of the differentiation therapy as an alternative approach to the treatment of rhabdomyosarcomas is suggested.

Introduction

The overproduction of tumour cells with their malignancy characteristics, in relation to the number of the cells that become differentiated, is the exaggerated peculiarity that distinguishes cancer from normal tissue.[1] Therefore, the origin and further progression of malignancy results from genetic changes that uncouple the normal balance between multiplication and differentiation so that there are too many growing cells.[2] The understanding of the molecular mechanisms that control growth and differentiation has allowed the development of specific differentiating drugs that lead to the elimination of tumorigenic cells both in vitro and in cancer patients.[3] This 'differentiation therapy', based on the malignant phenotype reversion toward a more physiological state, causes a re-entry of cells into the maturation pathway and a finite proliferative capability.

In comparison with the classical cytotoxic chemotherapy which present important side effects in patients, the use of low doses of antineoplastic drugs has proved to induce therapeutic differentiation in some human tumours,[4] and the synthesis of new differentiating drugs from well-known chemical structures has allowed lessen the adverse cytotoxicity.[5] The differentiation therapy has been successfully used in patients with blood-borne tumours and only a few number of studies have shown effectiveness in solid tumours.[6] Previously, uracil analogous,[7] new retinoid derivatives[3c] or butyric acid prodrugs[5] have been described to induce differentiation.

The non-naturally occurring base with known antitumor activity, 5-fluorouracil (5-FU), acts by a well-established mechanism.[8] Although it inhibits the key enzyme for DNA synthesis thymidylate synthetase, the false pyrimidine base is also incorporated into the RNA chain, making it unproductive.

The high toxicity of the 5-FU[9] has motivated an important research in order to find new less toxic molecules than 5-FU itself. Thus, many compounds including cyclic[10] Ftorafur and Doxofluridine, and acyclic[11] radicals attached to the 5-FU have been synthesized. The overall idea has been the obtaining of nucleoside sugar mimetics that make the structure able to inhibit the enzyme thymidylate synthetase or produce a prodrug of 5-FU.

In this sense, we have previously reported a series of 5-FU prodrugs with antitumor activity and low toxicity

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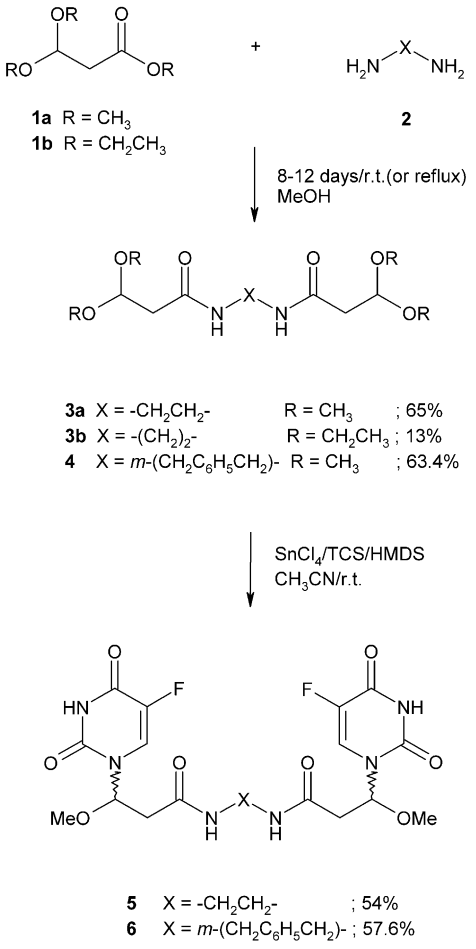
in vivo.[12] Moreover, these 5-FU derivatives induced myogenic differentiation in rhabdomyosarcoma human cells.[13,14] Our results showed that this type of tumour, (the most frequent soft tissue malignancy in paediatrics patients) may be amenable for differentiation therapy.

Prodrugs in which the 5-FU moiety has been attached to aminoacids[15] or even peptides[16] have also been reported. Finally, compounds bearing two or more molecules of 5-FU in their structures as well as 5-FU supported on a polymeric matrix have been also described.[17]

We herein report the design and synthesis of compounds with the following chemical characteristics (Scheme 1):

A. Two 5-FU moieties are attached as hemiaminal groups to the structures. These compounds would need in vivo activation to deliver 5-FU.[18]
B. A bridge-chain with two amide bonds between the two molecules of 5-FU. This spacer has been changed in length and nature in order to investigate size, lipophilicity, and conformational effects. The amide bond is an important constituent in many biologically active compounds and the preparation of substituted amides has received much attention. Some amide derivatives with 5-FU attached to them have been reported.[19]



Scheme 1.

The rationale for these compounds is based on the following aspects:

1. The possibility to release two moles of 5-FU per mole of the 'double-headed' *O*,*N*-acetal with the subsequent increase in activity, and
2. The chemical fragment bearing the 5-FU molecules might improve the pharmacokinetic characteristics of the cytostatic agent 5-FU.

In this report, the synthesized 5-FU derivatives are evaluated for their antitumor activity and their ability to induce myogenic maturation in an in vitro human rhabdomyosarcoma experimental model. Specific markers of normal muscular differentiation are investigated to determine if these compounds force the tumoral cells to re-entry into the normal cell cycle. Finally, possible implications of differentiation therapy in solid tumours are discussed.

Results

Chemistry

Compounds **5** and **6** were synthesized as described Scheme 1. The bis-acetal compounds **3** and **4** were obtained as described by Philips and Rae[20] for the synthesis of diamides. Hence, two equivalents of methyl 3,3-dimethoxypropionate (**1**) were stirred in a methanolic solution with one equivalent of the diamine **2**.

Other procedures for aminolysis of esters require high temperatures and/or long reaction times.[21] Some of the reported catalysts were not compatible with the structures in Scheme 1. The use of an ethyl ester as in **1b** to obtain **3b** lowered the yield.

In an attempt to improve the diamide formation the reaction was carried out using trimethylaluminum[22] as a catalyst, but the results were not satisfactory.

Products **5** and **6** were obtained by reaction of the appropriate bis-acetal with two equivalents of 5-FU, following a modification[11a] of the method described by Yasumoto.[23] Thus, hexamethyldisilazane (HMDS) and chlorotrimethylsilane (TCS) were added to a stirred suspension of 1 equiv of **3** and **4** and 2.5 equivalents of 5-FU in dry acetonitrile.

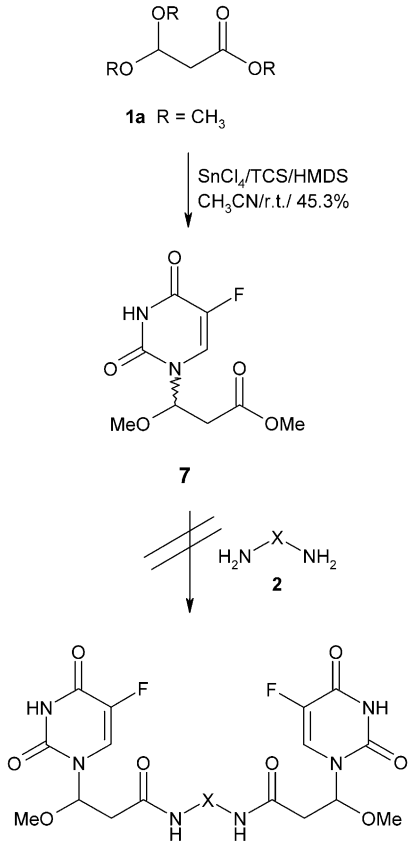
A solution of 4 equivalents of $SnCl_4$ in the same solvent was then added dropwise and the reaction was carried out at room temperature for 20 h. The reaction was quenched with $NaHCO_3$ and the crude was purified by flash chromatography to afford products **5** and **6** as a mixture of stereoisomers.

An alternative synthetic pathway to obtain compounds **5** and **6** was unsuccessful. Compound **7** was synthesized from ester **1a** (Scheme 2), using the above described conditions. In our hands the diamide formation starting from **7**, after the incorporation of the 5-FU moiety, did not improve the overall yields.

Mechanistically, the electrophilic attack of $SnCl_4$ on an oxygen atom of the bis-acetals (Fig. 1) generates an oxonium ion **8**. Subsequent nucleophilic addition of 2,4-bis(trimethylsilyl)-5-fluorouracil (TMS-FU) leads to the products. This attack occurs on both faces of **8**, which leads to a pair of enantiomers and one *meso* form, which were not separated.

Moreover, in all cases the attachment of the 5-FU moiety occurs at N^1—and never at N^3—position of the uracil ring. This is probably due to the steric hindrance caused by the two bulky silyloxy groups at C_2- and C_4-positions of the uracil ring.

These facts were confirmed through ^1H NMR studies. As established by Ozaki et al.,[24] the H_6 coupling constant value is indicative of the substitution pattern on the 5-FU moiety. Compounds **5** and **6** ^1H NMR agreed with a N^1-substitution on the 5-FU.



Scheme 2.

Thymidylate synthetase (TS) inhibition assays

The inhibitory activity of the compounds **5**, **6**, and **7** on the Thymidylate synthetase isolated from *Lactobacillus casei* (LcTS) has been tested. These compounds did not show any activity at a 500 μM concentration (Table 1) as expected since they do not have a ribose or a ribose mimetic bond to the 5-FU. Nevertheless, a 5-FU liberation could be explained in the presence of other enzymatic systems.[25]

In vitro growth rate inhibition

5-FU derivatives were tested in vitro against human rhabdomyosarcoma (RD) cell line. In comparison with the growth rate of control rhabdomyosarcoma cells all concentrations of the drugs—5-FU and derivatives—had a substantial inhibitory effect (Fig. 2). Inhibition was significantly greater in cells treated with 5-FU than in cultures treated with different drugs concentrations and the percentage of viable cells after treatment with 5-FU decreased for as long as 6 days to 70% (Fig. 2a). This indicates the cytotoxic effect of 5-FU on this type of tumour,[13] which was previously shown in other cell lines.[26] In contrast, the derivatives **5**, **6** and **7** caused a growth inhibition less marked and appeared later; the viability after treatment was >90%, similar to the control cultures (Fig. 2b). Moreover, the doses that inhibit the proliferation were more lower in all compounds

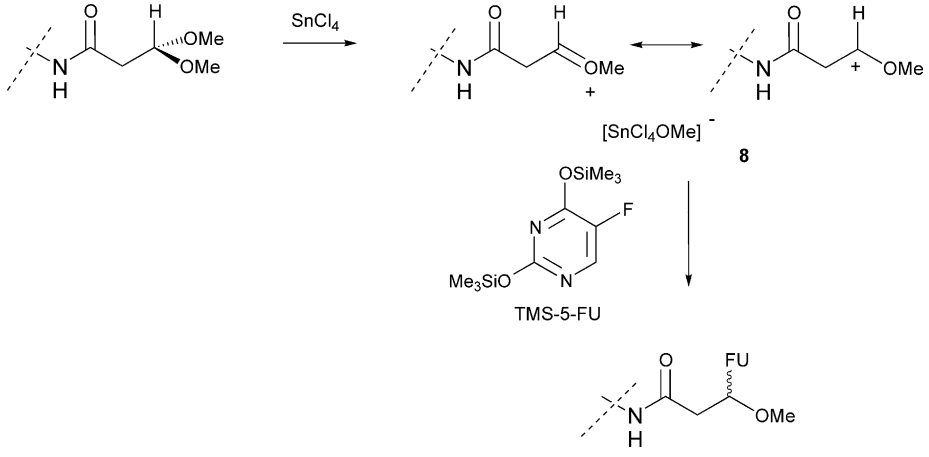


Figure 1. Electrophilic attack of $SnCl_4$ on an oxygen atom of the bis-acetals and nucleophilic addition of 2,4-bis(trimethylsilyl)-5-fluorouracil (TMS-FU).

Table 1. Percentage of inhibitory activity in μM against thymidylate synthetase

	Compounds inhibition (μM)	
5	600	NI
6	119	NI
7	652	16% I

NI, no inhibition; I, inhibition.

than in 5-FU. These data explain the lesser cytotoxic effect of the derivatives in comparison with 5-FU.

In vitro morphology

Phase-contrast microscopic observations showed that parental RD cells grew forming confluent aggregates of

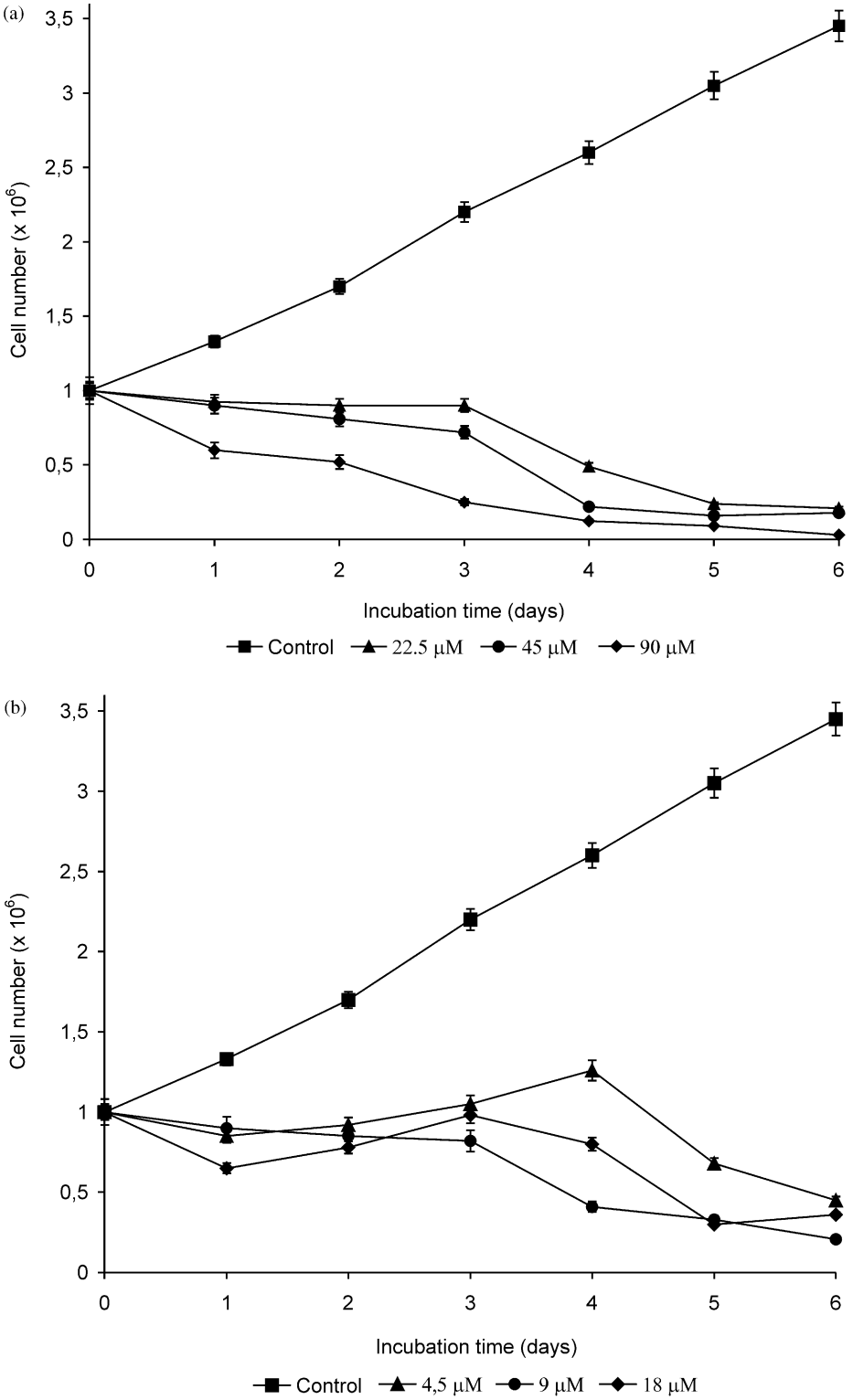
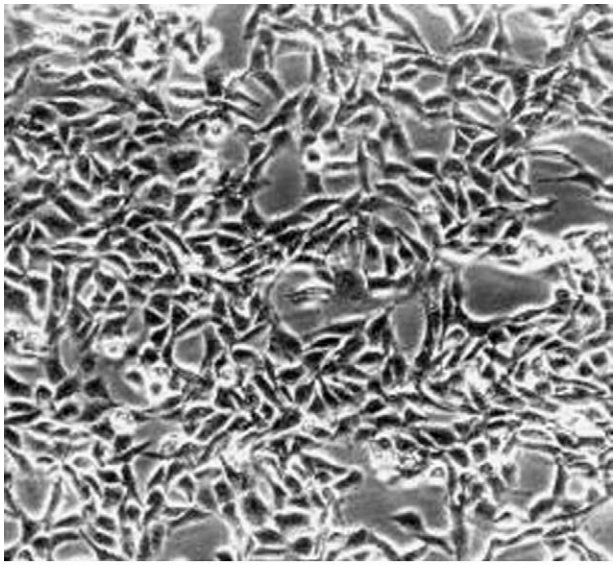


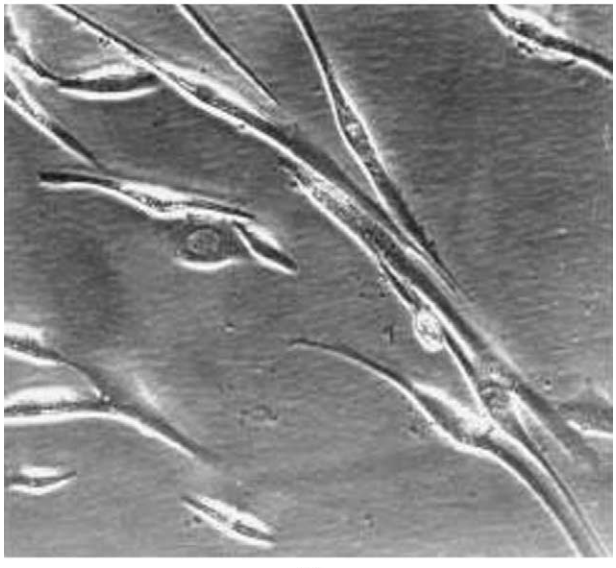
Figure 2. Effect of treatment with 5-FU (a) and **5** (b) on the growth rate of rhabdomyosarcoma RD cell line. Results are the means of three experiments conducted in triplicate; bars, SE.

rounded and polygonal cells, characterised by a single nucleus and scarce cytoplasm (Fig. 3a). However, after treatment during 6 days with the derivatives (4.5 and 9 μM **5**; 3.5 and 7 μM **6** or 25 μM **7**) the most of cell population grew in a parallel pattern and showed characteristics of adult muscle cells.[27] These elongated and multinucleated myotube-like giant cells represented up to 70% of the cell population in the cultures treated with **6** and **5** (Fig. 3b).

Rhabdomyosarcoma RD cells treated with 5-FU showed an increased proportion (up to 75%) of dead cells and a little number if myotube-like giant cells (7–10%).



(a)



(b)

Figure 3. Phase-contrast microscopy of human RD cells before and after 6 days of treatment with the compounds: (a) morphology of RD parental cell line that formed aggregates of small spindle-shaped and polygonal mononuclear cells (× 6); (b) elongated RD cells treated with the derivative 6 (7 μM) growing in parallel and forming myotube-like giant cells (× 6).

Modifications in intermediate filaments

The RD cells treated with the different doses of drugs modified the expression of desmin and vimentin proteins similar to the process of normal myogenesis[28] with a substitution of vimentin by desmin. Desmin protein is used as a differentiation marker in the developing,[29] whereas a largest vimentin expression is found in poorly differentiated and highly aggressive tumours.[30]

Thus, the quantitative analysis by FACs reported in Table 2 showed a significant increase in desmin expression, mainly with the doses of derivatives that induce the morphological changes. Compounds **5**, **6** and **7** significantly increased twice the quantity of desmin expression in comparison with the parental cells. However, the percentage of vimentin-positive cells decreased significantly after 6 days of treatment.

5-FU showed less significant changes in the degree of cytoskeletal organization that indicates its cytotoxic effect which hindered the re-entry in the program of muscle differentiation.[31]

Discussion

Neoplastic transformation is characterized by inappropriate cell proliferation and/or altered patterns of cell death. However, neoplastic transformation does not necessarily destroy the potential for expression of differentiated characteristics. The so-called differentiation therapy represents a viable option to control cancer growth and progression. As cytotoxicity is not the primary goal of this therapy, it is expected that this type of treatment will be more selective than conventional chemotherapy.

Compounds **5**, **6**, and **7** showed a remarkably low cytotoxic effect when compared to that using 5-FU itself. The high viability observed on RD cells after treatment

Table 2. Quantitative FACScan analysis of prodrugs and 5-FU effects on intermediate filaments

Compd	Concn (μM)	Desmin (%)	Vimentin (%)
Control		26.72±2.3	85.15±1.5
	4.5	56.74±1.2[a]	38.64±2.6[a]
5	9	42.43±2.4[b]	30.78±1.4[a]
	18	44.93±2.5[b]	40.31±0.7[a]
	3.5	66.38±3.2[a]	59.78±2.9[a]
6	7	78.95±1.3[a]	74.63±3.1
	14	33.21±1.7	31.14±2.6[a]
	12.5	33.61±0.9	39.1±1.7[a]
7	25	63.86±1.5[a]	81.2±3.4
	50	58.34±2.8[a]	75.86±3.2
	45	27.21±1.4	50.59±2.5[a]
5-FU	90	47.03±3.2[a]	75.56±2.6
	180	63.05±2.5[a]	83.39±1.7

All data are means±SEM of four independent determinations. Significance was determined by comparison of the means with Student's *t*-test.

[a]Significantly different ($p<0.001$) compared with parental RD.

[b]Significantly different ($p<0.05$) compared with parental RD.

with **5**, **6**, and **7** (>90%) is one of the biological characteristics for a potential drug to be used in the differentiation therapy.

Dimeric compounds **5** and **6** clearly displayed a better activity than monomeric compound **7**. An in vivo conversion into a monomer-like compound could not be precluded because of the amidic nature of their linker.

The control compound 5-FU showed a dose-dependence effect on desmin (Table 2) that was not observed in compounds **5**, **6**, and **7**. This could be a consequence of a diminished 5-FU in vivo released and it explains their lower cytotoxicity compared to 5-FU.

These compounds clearly behaved as differentiation inducers since the differentiation marker desmin increased in treated RD cells. The low vimentin expression found in treated RD cells is also indicative of a less aggressive tumour cell. This change correlates with the in vitro cell RD morphology as adult differentiated muscle cells were obtained.

Most important, this behaviour could not be compared with cells treated with 5-FU because of the increased amount of dead cells. Clearly, compounds **5**, **6**, and **7** are devoided of the cytotoxic effects of 5-FU mainly because they do not inhibit the enzyme TS, but they preserve its differentiation properties and hence they may be acting through the same mechanism as differentiators.

Conclusions

Five new bis 5-FU containing compounds have been synthesized and evaluated as antitumoral drugs. It has been shown that they induce differentiation with low cytotoxicity on a rhabdomyosarcoma cell line. Although more in vitro and in vivo studies will be necessary to determine whether they induce differentiation interacting at the same level as it does the 5-FU, our results suggest that compounds **5**, **6**, and **7** are less toxic than 5-FU itself and they might represent an important contribution to the differentiation therapy of solid tumours such as rhabdomyosarcoma.

Experimental

^{1}H (300.13 MHz) and ^{13}C (75.78 MHz) NMR spectra were recorded on a Bruker AM-300 spectrometer or a Bruker AM-400 spectrometer. Chemical shifts (δ) are given in parts per million (ppm) upfield from tetramethylsilane (TMS) as an internal standard. All products reported showed ^{1}H NMR and ^{13}C NMR spectra in agreement with the assigned structures. IR spectra (KBr pellets) were recorded on a Perkin-Elmer 782 spectrometer coupled to a 3600 Data Station. Melting points (mp) were determined on an Electrothermal Melting Point apparatus and are uncorrected. Elemental analyses were determined on a Perkin-Elmer 240C apparatus or Fisons Carlo Erba EA 1108.

Flash chromatography were performed on silica gel Merck 60 (230–240 mesh), using a 0.4 kg/cm pressure and MeOH/$CHCl_3$ as eluent. TLC was performed on Merck precoated 60 F_{254} plates and MeOH/$CHCl_3$ as eluent and were developed either on UV light at 254 nm or under hot H_2SO_4 steam. All compounds obtained commercially were used without further purification. Organic solutions were dried over anhydrous Na_2SO_4. all the anhydrous solvents were distilled over P_2O_5 prior to use.

N,*N*′-Bis-(3,3,dimethoxypropionyl)-ethylendiamine (3a)

A solution of methyl 3,3-dimethoxypropionate (4.7 mL, 33.1 mmol) and ethylenediamine (1.11 mL, 16.6 mmol) in absolute methanol (25 mL) was stirred at room temperature for 12 days. Partial evaporation under vacuum of the solvent gave crystalline **3a** (3.18 g, 65%): mp 135–137 °C. ^{1}H NMR ($CDCl_3$): δ 2.5 (4H, d, J = 5.3 Hz, CO–C$\underline{H}_2$), 3.35 (16H, s, OC$\underline{H}_3$ and N–C$\underline{H}_2$), 4.67 (2H, dd, J = 5.3 Hz, CH_2–C$\underline{H}$(OCH_3)$_2$) and 6.67 (2H, s, N$\underline{H}$). ^{13}C NMR ($CDCl_3$): δ 39.59 ($\underline{C}H_2$–N), 40.87 (CO–$\underline{C}H_2$–CH), 52.12 (O$\underline{C}H_3$), 102.15 ($\underline{C}H_2$–$\underline{C}$H(OCH_3)$_2$) and 170 ($\underline{C}$O). IR (KBr) υ: 3300 (s, N–H), 1646 (s, C=O). Anal. ($C_{12}H_{24}N_2O_6$): calcd C: 49.30%; H: 8.27%; N: 9.58%. Found. C: 49.30%; H: 8.28%; N: 9.65%.

***N*,*N*′-Bis-(3,3-diethoxypropionyl)ethylenediamine (3b).** A solution of ethyl 3,3-diethoxypropionate (7.1 mL, 33 mmol) and ethylenediamine (1.11 mL, 16.6 mmol) in absolute ethanol (30 mL), was stirred at room temperature for 11 days. Partially evaporation under vacuum of the solvent gave crystalline **3b** (0.76 g, 13%): mp 135–137 °C (see Results). ^{1}H NMR ($CDCl_3$): δ 1.16 (6H, dd, J = 7.0 Hz, O–CH_2–C$\underline{H}_3$), 2.49 (4H, d, J = 5.2 Hz, CO–C$\underline{H}_2$), 3.32 (4H, dd, J = 2.6 Hz, C$\underline{H}_2$–NH), 3.49 (4H, dq, J = 7.0, 9.2 Hz, O–C$\underline{H}_2$–CH_3), 3.64 (4H, dq, J = 7, 9.52 Hz, O–C$\underline{H}_2$–CH_3), 4.77 (2H, dd, J = 5.2 Hz, CH_2–C$\underline{H}$(O–CH_2– CH_3)$_2$) and 6.7 (2H, s, N$\underline{H}$). ^{13}C NMR ($\underline{C}DCl_3$): δ 15.26 (O–CH_2–$\underline{C}H_3$), 39.47 ($\underline{C}H_2$–N), 41.91 (CO–$\underline{C}H_2$–CH_3), 100.20 (CH_2–$\underline{C}$H(O–CH_2–CH_3)$_2$) and 170.14 ($\underline{C}$O). IR (KBr) υ: 3301 (m, N–H), 1641 (s, C=O). Anal. ($C_{16}H_{32}N_2O_6$): calcd C: 55.15%; H: 9.25%; N: 8.03%. Found. C: 54.60%; H: 9.08%; N: 8.57%.

***N*,*N*′ - Bis - (*3,3* - dimethoxypropionyl) - α,α′ - diamino - *p* - xylene (4).** A solution of methyl 3,3-dimethoxypropionate (4.8 mL, 33.8 mmol) and α,α′-diamino-*p*-xylene (2.29 g, 16.8 mmol) in absolute methanol (25 mL) was refluxed for 10 days. Then, the reaction mixture was diluted with water and extracted with $CHCl_3$ (7 × 20 mL). The organic layer was, separated, dried and concentrated under vacuum to give crystalline **7** (3.921 g, 63.4%): mp 148–149 °C. ^{1}H NMR ($CDCl_3$): δ 2.59 (4H, d, J = 5.3 Hz, CO–C$\underline{H}_2$), 3.40 (12H, s, O–C$\underline{H}_3$), 4.43 (4H, d, J = 5.8 Hz, Ar–C$\underline{H}_2$–NH), 4.73 (2H, dd, J = 5.3 Hz, CH_2–C$\underline{H}$(O–CH_3)$_2$), 6.56 (2H, bt, N$\underline{H}$) and 7.25 (4H, s, Ar–$\underline{H}$). ^{13}C NMR ($CDCl_3$): δ 41.0 ($\underline{C}$O–CH_2), 43.10 (Ar–$\underline{C}H_2$), 54.18 (O–$\underline{C}H_3$), 102.28 (CH_2–$\underline{C}$H(O–CH_3)$_2$), 127.85 ($\underline{C}$ Arom.), 137.60 ($\underline{C}$ Arom.) and 169.03 ($\underline{C}$=O). IR (KBr) υ: 3301 (s, N–H), 1634 (s, C=O), 830 and 785 (Arom. *p*-sustd). Anal.

($C_{18}H_{28}N_2O_6$): calcd C: 58.68%; H: 7.65%; N: 7.63%. Found. C: 58.66%; H: 7.64%; N: 7.57%.

General procedure for synthesis of 5-FU containing compounds 5–7

To a stirred suspension of appropriated bis-acetal **3a–4** (1.71 mmol) and 5-fluorouracil (4.27 mmol) in dry acetonitrile (20 mL), hexamethyldisilazane (HMDS, 0.689 g) and chlorotrimethylsilane (TCS, 0.462 g) were added. Then, a solution of $SnCl_4$ (6.84 mmol) in the same solvent (3 mL) was added dropwise. Stirring was continued for 20 h at room temperature, then neutralized with aqueous 4% $NaHCO_3$, the mixture filtered to remove inorganic salts and the solvent was evaporated to dryness under reduced pressure. The residue was subjected to flash chromatography ($MeOH/CHCl_3$, 1:12) to afford products **5** and **6** in 54 and 57.6% yields, respectively. All products gave satisfactory elemental analyses and spectroscopic data (^1H, ^{13}C NMR and IR). In a similar way was synthesized **7** from **1a**.

Product; yield; spectroscopic data:

***N,N′*-Bis-(3-methoxy-3-(5-fluorouracil-1-yl)propionyl)ethylenediamine (5).** 54%; ^1H NMR (DMSO-d_6): δ 2.52 (2H, dd, $J = 5.5$, 14.8 Hz, CO–C$\underline{H}_2$), 2.64 (2H, dd, $J = 7.3$, 14.8 Hz, CO–C$\underline{H}_2$), 3.01 (4H, bs, CH_2–C$\underline{H}_2$–NH), 3.19 (6H, s, O–C$\underline{H}_3$), 5.77 (2H, bdd, $J = 7.3$ Hz, CH_2–C$\underline{H}$), 7.87 (2H, d, $J = 6.8$ Hz, $\underline{H}_{5\text{-FU}}$), 7.93 (2H, s, N$\underline{H}$) and 11.6 (2H, s, N$\underline{H}_{5\text{-FU}}$). ^{13}C NMR (DMSO-d_6): δ 38.11 ($\underline{C}H_2$–NH), 40.12 (CO–$\underline{C}H_2$–CH), 55.94 (O–$\underline{C}H_3$), 83.70 (CH_2–$\underline{C}H$), 124.46 (d, $J = 33.3$ Hz, $\underline{C}_6$ 5-FU), 140.38 (d, $J = 23.18$ Hz, $\underline{C}_5$ 5-FU), 149.54 ($\underline{C}_2$ 5-FU), 157.11 (d, $J = 26.5$ Hz, $\underline{C}_4$ 5-FU) and 167.35 ($\underline{C}$=O). IR (KBr) υ: 3352 (s, C–H), 1740–1652 (s, C=O) and 1234 (s, C–F). Anal. ($C_{18}H_{22}F_2N_6O_8 \cdot 2.4H_2O \cdot 1.2$ MeOH): calcd C: 40.45%; H: 5.58%; N: 14.74%. Found: C: 40.55%; H: 4.84%; N: 14.17%.

***N,N′*-Bis-(*3*-methoxy-3-(5-fluorouracil-1-yl)propionyl)-α,α′-diamino-*m*-xylene (6).** 57.6%; ^1H NMR (DMSO-d_6): δ 2.62 (2H, dd, $J = 5.7$, 14.5 Hz, CO–C$\underline{H}_2$), 2.73 (2H, dd, $J = 7.1$, 14.5 Hz, CO–C$\underline{H}_2$), 3.21 (6H, s, O–C$\underline{H}_3$), 4.16 (2H, dd, $J = 5.7$, 15.3 Hz, Ar–C$\underline{H}_2$), 4.23 (2H, dd, $J = 5.9$, 15.3 Hz, Ar–C$\underline{H}_2$), 5.81 (2H, dd, $J = 5.7$ Hz, CH_2–C$\underline{H}$), 7.11 (4H, s, Ar–$\underline{H}$), 7.85 (2H, d, $J = 6.6$ Hz, $\underline{H}_{5\text{-FU}}$), 8.42 (2H, bt, CO–N$\underline{H}$) and 11.7 (2H, s, N$\underline{H}_{5\text{-FU}}$). ^{13}C NMR (DMSO-d_6): δ, 40.12 (CO–$\underline{C}H_2$–CH), 41.84 (Ar–$\underline{C}H_2$–NH), 56.03 (O–$\underline{C}H_3$), 83.78 (CH_2–$\underline{C}H$), 124.50 (d, $J = 33.4$ Hz, $\underline{C}_6$ 5-FU), 127.13 ($\underline{C}$ Arom.), 137.72 ($\underline{C}$ Arom.), 140.45 (d, $J = 23.2$ Hz, $\underline{C}_5$ 5-FU), 149.63 ($\underline{C}_2$ 5-FU), 157.26 (d, $J = 26.2$ Hz, $\underline{C}_4$ 5-FU) and 167.29 ($\underline{C}$=O). IR (KBr) υ: 3322 (m, C–H), 3072 (m, N–H), 1715–1669 (s, C=O) and 1258 (m, C–F). Anal. ($C_{24}H_{26}F_2N_6O_8 \cdot 1.5$ MeOH): calcd C: 49.99%; H: 5.26%; N: 13.71%. Found: C: 49.88%; H: 4.91%; N: 13.09%.

Methyl 3-methoxy-3-(5-fluorouracil-1-yl)propanoate (7). 45.3%; mp 150–152 °C; ^1H NMR (DMSO-d_6): δ 2.72 (1H, dd, $J = 5.38$, 15.74 Hz, CO–C$\underline{H}_2$), 2.79 (1H, dd, $J = 7.14$, 15.74 Hz, CO–C$\underline{H}_2$), 3.39 (3H, s, O–C$\underline{H}_3$), 3.7 (3H, s, COO–C$\underline{H}_3$), 5.95 (1H, dd, $J = 5.38$, 7.14 Hz, O–C$\underline{H}$–N), 7.4 (1H, d, $J = 5.7$ Hz, $\underline{H}_{5\text{-FU}}$), and 9.7 (1H, s, N$\underline{H}_{5\text{-FU}}$). ^{13}C NMR (DMSO-d_6): δ 39.61 ($\underline{C}H_2$–CO), 52.32 (O–$\underline{C}H_3$), 57.42 (COO–$\underline{C}H_3$), 84.04 (O–$\underline{C}H$–N), 122.77 (d, $J = 33.6$ Hz, $\underline{C}_6$ 5-FU), 141.18 (d, $J = 240.5$ Hz, $\underline{C}_5$ 5-FU), 149.48 ($\underline{C}_2$ 5-FU), 156.95 (d, $J = 26.7$ Hz, $\underline{C}_4$ 5-FU) and 168.59 ($\underline{C}$OOMe). IR (KBr) υ: 3350 (s, C–H), 1733–1645 (s, C=O) and 1235 (s, C–F). Anal. ($C_9H_{11}FN_2O_5$): calcd C: 43.90%; H: 4.50%; N: 11.37%. Found: C: 44.16%; H: 4.59%; N: 11.367%.

Biological Evaluation

Thymidylate synthetase (TS) inhibition assays

The enzyme *Lactobacillus casei* thymidylate synthetase (LcTS) has been extracted from yeast cells purified at electrophoretic homogenity as reported.[32] The activity of the enzyme has been tested spectrophotometrically by standard TS assay[33] following the increasing absorbance at 340 nm due to the 7,8-dihydrofolate formation under steady state kinetic conditions.

The compounds have been tested for their inhibitory properties against LcTS, spectrophotometrically by following the standard TS assays in presence of different concentrations of inhibitors. Owing to the low inhibitory activity of the compounds, only a percentage of inhibition is given.

Cell culture

The rhabdomyosarcoma cell line RD,[34] derived from a human embryonic rhabdomyosarcoma, was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). RD cells were routinely maintained in monolayer cultures at 37 °C in an atmosphere, containing 5% carbon dioxide with Dulbecco's modified Eagle medium (DMEM) (Gibco, NY, USA) supplemented with 10% foetal bovine serum (FBS) (Gibco), 20 $mmol\,L^{-1}$ L-glutamine, 3.5 mg μL^{-1} sodium bicarbonate, 4.5 g L^{-1} glucose, 250 U mL^{-1} ampicilin and 20 μg mL^{-1} streptomycin. The same batch of FBS was use for all experiments to obviate variations in quality.

Drug treatments

After synthesis and purification of **5**, **6**, **7** and 5-FU stock solutions were prepared. The drugs were dissolved in DMSO and stored at −20 °C. For each experiment, the stock solution was further diluted in medium to the desired concentration. The final solvent concentration in cell culture was less than 2% v/v of DMSO, a concentration without effect on cell replication. The cells were detached from the surface of the tissue culture flasks with phosphate-buffered saline (PBS)/EDTA (0.02%) and diluted in Dulbecco's medium to obtain cultures of 1×10^6 cells. RD cells were exposed continuously to three different drug concentrations: 4.5, 9 and 18 μM for 5, 3.5, 7 and 14 μM for 6, 12.5, 25 and 50 μM for 7, 45, 90 and 180 μM for 5-FU. Parallel cultures of RD cells in medium with DMSO were used as controls. The medium in both

control and drug-treated cultures was replaced every 48 h, and the cultures were maintained and examined every 24 h for 6 days. All experiments were performed in triplicate and repeated twice.

Growth-inhibition assays

Culture flask (25 cm^2) (Greiner, Nürtingen, Germany) containing parental RD cells (1×10^6) were exposed to growth medium supplemented with the different concentrations of compounds. As a control, culture flasks were seeded with cells in standard growth medium with DMSO. In each experiment, cells from the culture flasks with each different drug concentration, and flasks with cells growing in drug-free medium, were harvested separately every 24 h for 6 days. Cell viability was assayed by the trypan blue exclusion test. The number of cells harvested was determined in a model ZBI Kontron Coulter counter.

In vitro morphology

RD cells were observed with an inverted light microscope before and after treatment with the drugs. Optic phase-contrast photographs were taken with a Nikon TM Phase Contrast-2, ELWD 0.3 inverted microscope. The number of myotube-like giant cells was counted by phase-contrast microscopy at intervals of 24 h. Cells that contained three or more nuclei were classified as myotube-like giant cells.

Fluorescence-activated cell sorting (FACScan)

In brief, 10^6 controls and RD treated cells for 6 days were transferred to universal screw cap tubes containing sterile PBS, then washed and centrifuged at 225*g* for 5 min. The supernatant was discarded, and the washing and centrifugation steps were repeated once or twice. The cells were permeabilized with methanol for 10 min, then washed three times in PBS and once in distilled water. The cells were incubated for 30 min at 4 °C with the anti-desmin (dilution 1:500) and anti-vimentin (dilution 1:200) monoclonal antibodies (mAbs) (Sigma, St. Louis, MO, USA), then washed twice in cold PBS and reincubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Sigma) at a dilution of 1:50 for FACS analysis.

Statistical analyses

The data derived from growth curves were subjected to analysis of variance with two independent factors. Student's *t*-test was used to analyse the differences between control and treated RD cells after FACS analysis. All data are means ± SEM of three separate experiments, and a p value less than or equal to 0.05 was considered significant.

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