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FATTY ACID CONJUGATES OF 2'-DEOXY-5-FLUOROURIDINE AS PRODRUGS FOR THE SELECTIVE DELIVERY OF 5-FLUOROURACIL TO TUMOR CELLS

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Abstract—We have prepared a novel class of prodrugs by coupling 2'-deoxy-5-fluorouridine (5dFU) to oleic (18:1) and docosahexaenoic (22:6) acids, respectively. The cytotoxic activity of the drug and its conjugates (5dFU–18:1 and 5dFU–22:6) has been assayed *in vitro* upon HT-29, a colon carcinoma cell line of human origin. After short term (2-hr) treatments with the drugs, both fatty acid conjugates of 5dFU showed cytotoxic activity in a dose-dependent way, while 5dFU alone was devoid of toxic effects within the whole range of concentrations (10–200 μM) tested. Following long term (24- or 48-hr) incubations only a fraction of the HT-29 cell population was sensitive to 5dFU, the rest of the population being resistant even at the highest concentration tested (200 μM). In contrast, 5dFU–oleic acid and, particularly, 5dFU–docosahexaenoic acids appeared toxic for the whole population of HT-29 cells under the same experimental conditions. The considerable gain in cell toxicity and, to a lesser extent, in selectivity resulted from the conjugation since the toxic effect of the drug alone was not modified when equimolar mixtures of 5dFU and fatty acids were assayed. These results confirm a previous study on the cytotoxicity of fatty acid derivatives of chlorambucil toward malignant lymphoblastoid cells and reinforce the potential use of fatty acid conjugates as efficient anti-tumor prodrugs.

A major problem in cancer chemotherapy is the lack of sufficient tumor selectivity of cytotoxic drugs. Many attempts have been made to increase the selectivity of chemotherapeutic agents by conjugating them with targeting macromolecules such as DNA, enzymes and antibodies raised against cancer-specific surface antigens, or by entrapping them in liposomes conjugated with antibodies [1–5].

As part of our program on the development of cancer-oriented drugs, we have initiated the synthesis of carrier-bound ketonucleosides attached by spacers of various length and functional groups to targeting macromolecules [6] and this work has been extended to the preparation of fatty acid conjugates of cytotoxic drug.

Previous work from our laboratory has provided evidence that: (a) the entry of fatty acids into cells is regulated and facilitated by α-fetoprotein (AFP†) through its interaction with specific receptors [7–9] and (b) an AFP/AFP-receptor pathway may be activated in neoplastic cells of varied origin as well as in normal lymphocytes undergoing blastic transformation [10–12].

On the basis of these observations we have, recently, explored the possibility of improving the efficiency of conventional antitumor drugs by the use of their fatty acid conjugates on neoplastic cells expressing AFP and AFP receptors. Covalent conjugates of chlorambucil and fatty acids of different degrees of unsaturation were prepared and assayed *in vitro* to check their cytotoxic activity against two lymphoma cell lines and comparatively quiescent and mitogen-activated normal human lymphocytes. The results obtained [13] showed that the coupling of chlorambucil with polyunsaturated fatty acids improves its *in vitro* toxicity as well as its selectivity against malignant lymphocytes compared with quiescent non-proliferating cells.

In the present study, we have extended the same approach to another class of antitumor drugs, derivatives of 2'-deoxy-5-fluorouridine (5dFU), and to tumor cells of epithelial origin which are by far those with the highest incidence among human individuals. Covalent complexes of 5dFU with oleic (18:1) and docosahexaenoic acid (22:6) were prepared and assayed upon HT-29 human colon carcinoma cells.

MATERIALS AND METHODS

General chemical methods

All reactions were conducted under nitrogen. Solutions were concentrated to dryness under reduced pressure below 30°. TLC was performed on Silica Gel 60 F_{254} (Merck, Darmstadt, F.R.G.) and Silica Gel 60 (230–400 mesh) was used for flash chromatography [14], with ethylacetate (A) and 1:1 ethylacetate–hexane (B). Nucleoside derivatives were detected by examination under UV light and unsaturated fatty acids were located by exposure to iodine vapours. R_f values are given for solvent B as the eluent. UV spectra were recorded with a Varian

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† Abbreviations: 5dFU, 2'-deoxy-5-fluorouridine; AFP, α-fetoprotein; DMAP, dimethylaminopyridine; DMEM, Dulbecco's modified Eagle's minimal essential medium; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin.

UV–VIS M 635 spectrophotometer and optical rotations were determined with a Roussel-Jouan Quick polarimeter. ^{1}H-NMR spectra were recorded for solutions in $CDCl_3$ with tetramethylsilane as the internal standard, using a Bruker MSL 300 spectrometer.

2'-Deoxy-5'-O-triphenylmethyl 5-flurouridine (**1**). A mixture of 5-dFU (123 mg, 0.5 mmol), dimethylaminopyridine (DMAP) (3 mg, 0.025 mmol), triethylamine (153 μL, 1.1 mmol), and chlorotriphenylmethane (153 mg, 0.55 mmol) in dry dichloromethane (5 mL) was stirred overnight at room temperature. The solution was concentrated and the residue was purified by flash chromatography (continuous gradient from dichloromethane to solvent A) to give crystalline **1** (203 mg, 83%) which, after recrystallization from dichloromethane–hexane, had m.p. 116–117°, $[\alpha]_D^{20}$ + 47.5° (*c* 0.2, chloroform), λ_{max} 270 nm (ε 8188), R_f 0.460 (Solvent A); lit. [15] m.p. 116°.

3' - O - t - Butyldimethylsilyl - 2' - deoxy - 5' - O - triphenylmethyl-5-fluorouridine (**2**). To a solution of **1** (190 mg, 0.389 mmol) in dry dimethylformamide (780 μL) imidazole (64 mg, 0.93 mmol) and *t*-butyldimethylsilyl chloride (70 mg, 0.467 mmol) were added. The mixture was stirred for 5 hr at room temperature, then concentrated. The residue in dichloromethane was washed with water, dried and concentrated. Flash chromatography (continuous gradient from hexane to solvent B) of the residue gave **2** (194 mg, 83%) which, after recrystallization from light petroleum ether, had m.p. 145°, $[\alpha]_D^{20}$ + 43° (*c* 0.2, chloroform), λ_{max} 270 nm (ε 8728), R_f 0.461 (solvent B).

3' - O - t - Butyldimethylsilyl-2'-deoxy-5-fluorouridine (**3**). A solution of **2** (182 mg, 0.302 mmol) in ethylacetate (665 μL) and aqueous 90% formic acid (1 mL) was stirred at room temperature for 1 hr, then diluted with ethylacetate (20 mL), and washed with half-saturated aqueous NaCl, saturated $NaHCO_3$ and water. The organic layer was dried and concentrated. Flash chromatography (continuous gradient from dichloromethane to solvent A) of the residue gave compound **3** (77 mg, 71%) which, after recrystallization from ethylacetate-hexane, had m.p. 171–172°; $[\alpha]_D^{20}$ + 37° (*c* 0.2, chloroform), λ_{max} 270 nm (ε 7940), R_f 0.235 (solvent B).

3' - O - t - Butyldimethylsilyl - 2' - deoxy - 5' - O - (9 - oct-adecenoyl)-5-fluorouridine (**4**). To a stirred solution of **3** (34 mg, 0.094 mmol) in dry dichloromethane (425 μL) were added DMAP (0.6 mg, 0.005 mmol), oleic acid (28 mg, 0.099 mmol) and dicyclohexylcarbodiimide (21 mg, 0.104 mmol). The mixture was kept for 1.5 hr at room temperature and then filtered. The filtrate was diluted with dichloromethane and washed with dilute acetic acid and water, dried and concentrated. Flash chromatography (continuous gradient from hexane to solvent B) of the residue gave the amorphous oleic ester **4** (55 mg, 94%), $[\alpha]_D^{20}$ + 35° (*c* 0.2, chloroform), λ_{max} 270 nm (ε 8907), R_f 0.541.

3' - O - t - Butyldimethylsilyl-2'-deoxy-5'-O-(4,7,10,13,16,19-docosahexaenoyl)-5-fluorouridine (**5**). Similar treatment of **3** (38 mg, 0.105 mmol) with docosahexaenoic acid afforded compound **5** (58 mg, 83%) as an amorphous product $[\alpha]_D^{20}$ + 29° (*c* 0.2, chloroform), λ_{max} 270 nm (ε 8380), R_f 0.55.

2'-Deoxy-5'-O-(9-octadecenoyl)-5-fluorouridine (**6**). Compound **4** (55 mg, 0.088 mmol) in dry tetrahydrofuran (2.2 mL) was treated with anhydrous tetrabutylammonium fluoride (76 mg, 0.29 mmol) and acetic acid (9.37 μL, 0.164 mmol) for 3.5 hr at room temperature. The solution was then concentrated and flash chromatography of the residue yielded crystalline **6** (41 mg, 91%), m.p. 90–92°. $[\alpha]_D^{20}$ + 23.6° (*c* 0.2, chloroform), λ_{max} 269 nm (ε 7759), R_f 0.107.

2' - Deoxy - 5' - O - (4,7,10,13,16,19-docosahexaenoyl)-5-fluorouridine (**7**). Similar treatment of **5** (58 mg, 0.087 mmol) afforded compound **7** (41 mg, 85%) as a waxy product, $[\alpha]_D^{20}$ + 14.5° (*c* 0.2, chloroform), λ_{max} (269.5 nm (ε 8750), R_f 0.108.

Cell cultures

HT-29 cells from the parental line were obtained from Dr Zweibaum (INSERM U-178, Villejuif, France). Cells were usually cultivated in Dulbecco's modified Eagle's minimal essential medium (DMEM, Eurobio, Paris, France) containing 4.5 g/L (2.5 mM) of glucose and supplemented with 5 or 10% fetal calf serum (Gibco, Grand Island, NY, U.S.A.), 2 mM glutamine and antibiotics. Cells were seeded in 96-well plates (Nunc, Intermed, France) at 6000 cells/well and cultivated at 37° in a humidified atmosphere of 95% air/5% CO_2. Cytotoxic assays were carried out after 48 hr of culture.

Peripheral blood mononuclear cells (PBMC) were isolated from blood of normal donors by gradient centrifugation in Ficoll-Paque (Pharmacia, Uppsala, Sweden) as described previously [16]. After the isolation, cells were cultured in complete RPMI at a cell density of 1×10^6 cell/mL. Viability was controlled by Trypan blue exclusion. Non-activated PBMC were incubated with drug as soon as they were isolated. Activated PBMC (T-lymphocytes) were incubated with drug after 48 hr in complete RPMI containing 2 μg/mL phytohemagglutinin (PHA-M, Sigma Chemical Co., Poole, U.K.).

Toxicity assays

Just before use, 5dFU or 5dFU–fatty acid complexes: 5dFU–oleic acid (5dFU-18:1) and 5dFU–docosahexenoic acid (5dFU–22:6) were dissolved in 96% ethanol and aliquots of these solutions were added to complete culture medium. In all cases, final ethanol concentration in the medium was 0.4% (v/v).

HT-29 cells, non-activated and PHA-activated PBMC were incubated for 2, 24 or 48 hr with concentrations ranging between 10 and 200 μM of drug or drug–fatty acid complex as well as of fatty acid or equimolar mixtures of drug and fatty acid. After incubation, the medium was discarded, the cells were washed three times in PBS and, if necessary, allowed to proliferate for up to 24 or 48 hr in the same volume of fresh, complete DMEM for HT-29 cells or RPMI containing 2 mg/mL PHA for PBMC. The latter assay evaluates, in fact, the capability of drug-heated PBMC to undergo mitogen-mediated blastic transformation. Control cells (HT-29 and PBMC), were cultured for the same period

of time in the corresponding complete, drug-free medium containing 0.4% ethanol (v/v). In all cases the viability of cells was estimated by the colorimetric proliferation test of Mosmann [17] 24 or 48 hr from the onset of drug treatment.

Results are expressed in terms of cellular growth (% of the control) (C.G.) calculated as the number of viable cells from the 550 nm absorbance values obtained:

G.I. = (Control cells − Drug-incubated cells/ Control cells) × 100
C.G. = 100 − G.I.

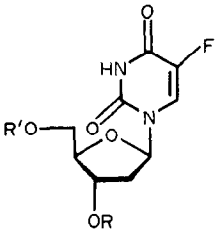
where G.I. is growth inhibition (% of the control).

RESULTS AND DISCUSSION

Chemistry

Treatment of 5dFU with chlorotriphenylmethane in the presence of triethylamine and a catalytic amount of DMAP [18] gave the 5′-tritylated compound (**1**) in 83% yield, together with a small amount of 3,5′- and 3′,5′-ditrityl derivatives. The ^{1}H-NMR spectrum of **1** contained, *inter alia*, a narrow doublet for H-3 at δ 9.23 ($J_{H,F}$ 4.44 Hz), a 1-proton doublet for H-6 at δ 7.81 ($J_{H,F}$ 5.96 Hz), a 15-proton multiplet for the aromatic protons of the trityl group centered at δ 7.33 and a 1-proton triplet for H-1′ at δ 6.29.

Reaction of **1** with *t*-butyldimethylsilyl chloride in the presence of imidazole afforded the 3′-O-silylated derivative **2** as a crystalline compound.



1 R = H, R′ = triphenylmethyl
2 R = BDMS, R′ = triphenylmethyl
3 R = BDMS, R′ = H
4 R = BDMS, R′ = oleoyl
5 R = BDMS, R′ = docosahexaenoyl
6 R = H, R′, = oleoyl
7 R = H, R′ = docosahexaenoyl
BDMS = *t*-butyldimethylsilyl

Selective removal of the trityl group with 90% formic acid [19] yielded 71% of 3′-*O*-*t*-butyldimethylsilyl-5dFU (**3**) as a crystalline product. The ^{1}H-NMR spectrum of **3** was essentially the same as that of **1** except for the absence of the aromatic protons of the trityl group and the presence of two singlets for the *t*-butyldimethylsilyl group at δ 0.89 and 0.09.

Treatment of **3** with various fatty acids in the presence of dicyclohexylcarbodiimide and DMAP [20] gave good yields of the oleic and docosahexaenoyl esters **4** and **5**, respectively. The ^{1}H-NMR spectra of **4** and **5** contained, *inter alia*, signals for the olefinic protons of the fatty acid centered at δ 5.34. In addition, the signals for H-5′$_{a,b}$ appeared markedly downfield at δ 4.43 and 4.27, respectively, confirming the structure assigned.

Removal of the silyl group with anhydrous tetrabutylammonium fluoride afforded good yields of the desired fatty acid esters **6** and **7**.

Toxicity against HT-29 cells

Figure 1 illustrates the results obtained with HT-29 cells incubated for 2 hr with 5dFU and 5dFU–fatty acid complexes, followed by 24 hr (A) or 48 hr (B) culture in drug-free medium. Under these conditions, no significant toxicity was observed towards cells incubated with the drug alone. Incubations with oleic or docosahexaenoic acid alone or with mixtures of the drug and oleic or docosahexenoic acid also lacked toxicity (not shown). However, both 5dFU–18:1 and 5dFU–22:6 complexes showed cytotoxic activity at all the concentrations tested. Fifty per cent inhibition of the

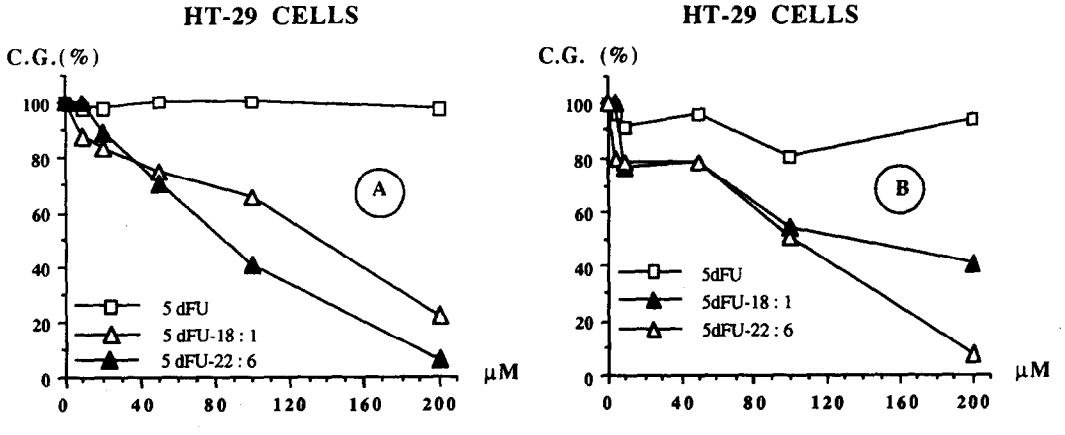


Fig. 1. Cytotoxic effect of several concentrations of 5dFU and conjugates of 5dFU with oleic acid (5dFU–18:1) and with docosahexaenoic acid (5dFU–22:6) on HT-29 cells after 2 hr of incubation with the drugs followed by 24 hr (A) or 48 hr (B) culture in drug-free complete DMEM medium. The cells were then subjected to the proliferation test of Mosmann [17]. The cellular growth (C.G., % of control) was calculated from these data. Data are the means of two independent experiments and the determinations were made in quadruplicate. SD was less than 20% of the mean.

Table 1. Concentrations (μM) of 5dFU, 5dFU-18:1 and 5dFU-22:6 necessary to cause a 50% inhibition of the growth of different control cell types assayed, at the time indicated and under different experimental conditions

	5dFU (μM)				5dFU-18:1 (μM)				5dFU-22:6 (μM)			
Treatment (hr)*	2		24	48	2		24	48	2		24	48
Time of culture (hr)†	24	48	24	48	24	48	24	48	24	48	24	48
HT-29 cells	∞	∞	∞	10	140	150	56	10	85	100	30	10
PBMC (quiescent)		∞				∞				130		
PBMC (activated)		∞				95				60		

* Treatment, time of incubation in the presence of the drug.

† Time after seeding at which aliquots of cultured cells were subjected to the proliferation test of Mosmann [17].

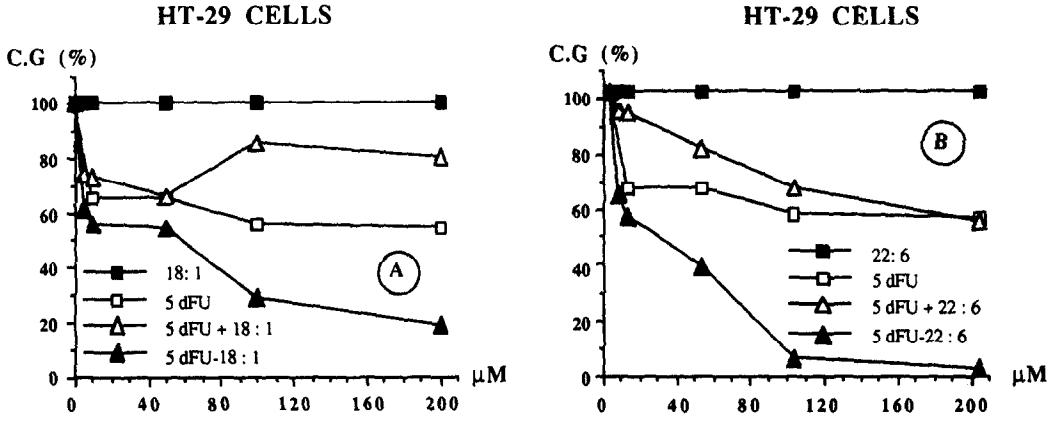


Fig. 2. Cytotoxic effect on HT-29 cells after 24 hr incubation with (A) several concentrations of oleic acid (18:1), 5dFU, conjugates of 5dFU with 18:1 (5dFU-18:1) and mixtures of 5eFU and 18:1 (5dFU + 18:1) and (B) several concentrations of docosahexaenoic acid (22:6), 5dFU, conjugates of 5dFU with 22:6 (5dFU-22:6) and mixtures of 5dFU and 22:6 (5dFU + 22.6). The cells were subjected to the proliferation test of Mosmann [17] immediately after 24 hr incubation with the drugs in complete DMEM medium. The cell growth (C.G., % of the control) was calculated from these data. Data are the mean of two or three independent experiments and the determinations were made in quadruplicate. SD was less than 20% of the mean.

cellular growth of control cells (IC_{50}) was attained at micromolar concentrations of 140 for 5dFU-18:1 and 85 for 5dFU-22:6, after 24 hr culture (Fig. 1A and Table 1). Similar values were observed after 48 hr culture (Fig. 1B and Table 1).

Somewhat different results were obtained when cells were treated for 24 hr instead of 2 hr (Fig. 2). Either 5dFU, 5dFU-18:1 or 5dFU-22:6 inhibited cell growth by 35–45% at concentrations as low as 10 μM. Further augmentation, up to 200 μM, improved the cytotoxic capability of 5dFU-18:1 and of 5dFU-22:6 while that of 5dFU alone remained unchanged. The respective curves of cell growth show two distinct slopes which probably reflect some cellular heterogeneity and suggest the presence of a cell population highly resistant to 5dFU alone. More evidence for this was obtained when the cultures were treated for 48 instead of 24 hr. Under these conditions 50% inhibition of growth occurred in the presence of a 10 μM concentration of either 5dFU, 5dFU-18:1 or 5dFU-22:6 (Fig. 3 and Table 1) and the IC_{50} remained unchanged with a further increase in the concentration of 5dFU or equimolar mixtures of 5dFU with fatty acids. In contrast, fatty acid conjugates of 5dFU become progressively more cytotoxic with increasing dose. This effect was particularly noticeable with 5dFU-22:6 complex (Figs 2B and 3B). Incubations with fatty acids alone were without effect.

Toxicity against human lymphocytes

The experiments illustrated in Fig. 4 and compiled in Table 1 were carried out in order to check the cytotoxic activity of 5dFU and its fatty acid conjugates

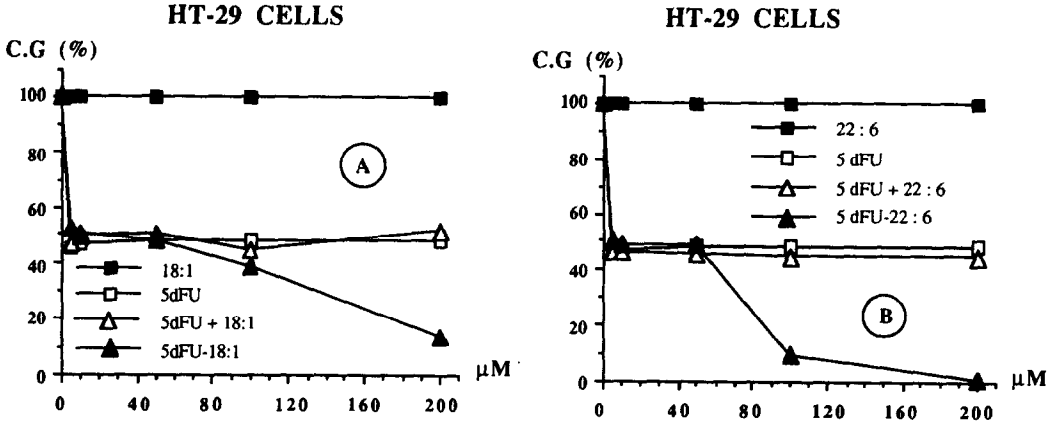


Fig. 3. Cytotoxic effect on HT-29 cells of (A) several concentrations of oleic acid (18:1), 5dFU, conjugates of 5dFU with 18:1 (5dFU–18:1) and mixtures of 5dFU and 18:1 (5dFU + 18:1) and (B) several concentrations of docosahexaenoic acid (22:6), 5dFU, conjugates of 5dFU with 22:6 (5dFU–22:6) and mixtures of 5dFU and 22:6 (5dFU) + 22:6). The cells were subjected to the proliferation test of Mosmann [17] after 48 hr incubation with the drugs in complete DMEM medium. The cell growth (C.G., % of the control) was calculated from these data. Data are the mean of two or three independent experiments and the determinations were made in quadruplicate. SD was less than 20% of the mean.

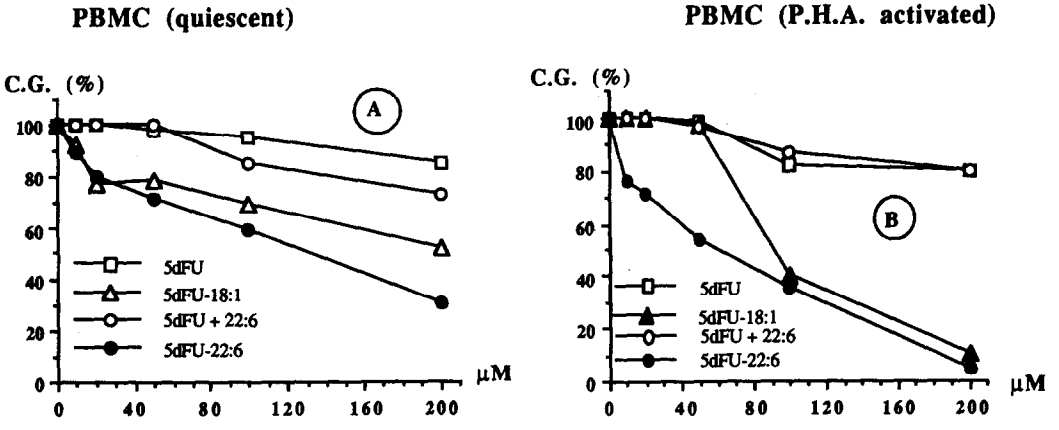


Fig. 4. Cytotoxic effect on quiescent (A) and PHA-activated (B) PBMC after 2 hr incubation in several concentrations of 5dFU and conjugates of 5dFU with oleic acid (5dFU–18:1) and docosahexaenoic acid (5dFU–22:6), as well as in mixtures of drug and fatty acids (5dFU + 18:1; 5dFU + 22:6), followed by 48 hr of culture in drug-free complete RPMI medium supplemented with PHA (2 μg/mL). Cells were then subjected to the proliferation test of Mosmann [17]. The results are the average of PBMC from three blood donors and determinations were made in quadruplicate. SD was less than 20%.

on normal, quiescent and PHA-activated human PBMC. The results obtained show the low toxicity of 5dFU and of equimolar mixtures of the drug with oleic (5dFU–18:1) and docosahexaenoic acid (5dFU–22:6) throughout the range of concentrations used. This is evidenced by the good capability of PBMC, either before or after 48 hr stimulation with PHA, to undergo normal activation. On the other hand, progressive cytotoxic effect was observed with increased concentrations of both 5dFU–18:1 and 5dFU–22:6. IC_{50} values (Table 1) were, nevertheless, higher for quiescent than for PHA-activated PBMC. The same difference was observed after 24 hr drug treatment (data not shown).

General comments

The first point which deserves attention from the results presented here is the considerable gain in cell toxicity of 5dFU when the drug is conjugated with fatty acids. This conclusion was already drawn with conjugates of fatty acids and chlorambucil using malignant lymphoblastoid cells as target elements [13]. The high toxicity observed after short term treatment with fatty acid–5dFU conjugates clearly

resulted from the conjugation since, taken separately, the drug, the fatty acids and their mixtures failed to produce any toxic effect at the same dose level, demonstrating the importance of the covalent linkage between 5dFU and fatty acids. 5dFU was toxic only after long term incubations (24 or 48 hr). Whether the elevated toxicity of the conjugates is due to an increased cell uptake, relative to the drug alone, or results from a slowed down metabolism of the drug in its conjugated form remains to be elucidated and is, at present, the object of further investigation. Another point of interest is the variable sensitivity of HT-29 cells to increased concentrations of drug or fatty acid–drug conjugates as evidenced by the two-slope curves of Fig. 3. A fraction of the HT-29 cell population appears highly sensitive to low doses (10 μM) of either 5dFU or its conjugates with 18:1 and 22:6. Further increase in drug concentration reveals, however, a second population resistant to 5dFU alone but sensitive to the cytotoxic effect of conjugates. These observations may be related to the pluripotent nature of the parent HT-29 cell line used here and confirmed by its capacity to induce tumors displaying different phenotypic characteristics. The above results suggest the use of 5dFU–unsaturated fatty acid conjugates in the therapy of 5dFU-resistant colon carcinomas.

Although both 5dFU–18:1 and 5dFU–22:6 were toxic against quiescent and mitogen-activated PBMC (Fig. 4 and Table 1), the latter were more sensitive to the conjugates than the former. In fact, the difference was underestimated since it is known that a variable proportion of PBMC (T and B cells, monocytes/macrophages) isolated from healthy blood donors are already activated before PHA stimulation. Therefore, fatty acid–5dFU conjugates possess some selectivity toward proliferating cells and could allow the finding of adequate conditions to assure a good killing efficiency against neoplastic cells while preserving sufficiently the pool of normal, resting immunocompetent cells.

The mechanism of entry of fatty acids into living cells has been extensively studied. Although still not unanimously accepted, the statement that the transfer of fatty acids to cells is mediated by membrane receptors specific for fatty acids and for their serum carrier proteins (albumin and AFP) is gaining general acceptance [21]. Work from our laboratory has provided experimental support of AFP-mediated facilitation of fatty acid transfer to cells expressing AFP receptors [7–9]. HT-29 cells [12], and other neoplastic cells of varied origin [8], contrary to their normal, quiescent counterparts, express AFP receptors and synthesize the protein. An explanation of the higher cytotoxicity associated with fatty acid–drug conjugates, relative to the drugs alone, may be the enhanced uptake of conjugates by cells expressing AFP and fatty acid receptors. Further investigations are, nevertheless, necessary to give experimental support to this hypothesis.

The results obtained previously by Anel *et al.* [13] with chlorambucil–fatty acid conjugates acting on human lymphoblastoid cells and those reported here with fatty acid complexes of 5dFU upon human colon carcinoma cells fit well with the hypothesis outlined above and encourage further work to assess the therapeutic potential of these prodrugs.

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