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Original article

Design, synthesis and *in vitro* drug release investigation of new potential 5-FU prodrugs

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ABSTRACT

In order to identify new efficient prodrugs of 5-fluorouracil (5-FU) and to develop an original targeting approach using 2-fluoro-2-deoxyglucose (FDG) as a potential drug carrier, eight original 5-FU derivatives were synthesized: 5-FU was attached by the N1 position of the pyrimidinic ring to the C1 position of the FDG structure either by direct coupling (**2a**) or *via* various spacers (**3**, **6a**–**c**, **10b** and **19**). A new sensitive high-performance liquid chromatography method was developed to simultaneously quantify 5-FU and its derivatives in human plasma and other relevant media at physiological temperatures. Half-lives were determined from the degradation profiles of these conjugates. Slow degradation of compounds **2a**, **3**, **10b** and **19** was observed *in vitro* at 37 °C, but no 5-FU release was noticed. By contrast, the *in vitro* drug release profiles of compounds **6a**–**c** followed pseudo-first-order kinetics, and 5-FU was found in all the media. The antiproliferative activity of the eight compounds was assessed *in vitro* by a fluorometric assay against two human solid cancer cell lines and one healthy cell line. A correlation was found between the activities of the compounds and their ability to release 5-FU efficiently.

1. Introduction

5-Fluorouracil (5-FU; Fig. 1) was introduced as a rationally designed anti-cancer agent more than fifty years ago [1]. It is widely used in the treatment of solid malignant tumors such as colorectal cancers. However, 5-FU displays various toxicities owing to its nonspecific cytotoxicity for tumor cells, loses efficiency through poor distribution to tumor sites, and is seriously limited in effectiveness by drug resistance. Despite some improvements in response rates achieved by combining 5-FU with newer chemotherapies such as irinotecan and oxaliplatin, new therapeutic strategies that directly act on the 5-FU activity are still needed [2,3].

Over the years, a better understanding of its mechanism of action has led to the development of several strategies for increasing the anti-cancer activity of 5-FU: to decrease its rapid metabolism, to increase its conversion into active metabolites, to maintain high concentrations in both blood and tumor over long periods of time, and more recently to identify predictive biomarkers of response to 5-FU-based chemotherapy [4].

Numerous derivatives have been synthesized to improve the physicochemical, biopharmaceutical and pharmacokinetic properties of 5-FU. Some of these have found a place in clinical practice, their main benefit being for oral administration. For example, capecitabine is an oral prodrug that allows higher intratumoral levels of 5-FU to be reached through tumor-specific bioconversion [5,6].

The control of drug level at the tumor site is a major factor on which the efficiency of cancer chemotherapy directly depends [7–11]. To obtain selective delivery of 5-FU to a tumor site, most attempts have focused on the development of new drug delivery systems based on passive targeting strategies [12]. To the best of our knowledge, selective delivery of 5-FU toward tumor sites using an active targeting strategy has never been reported, except with the ADEPT strategy (antibody-directed enzyme prodrug therapy) [13,14].

Active tumor-targeting strategies have been explored by our team for several years using various vectors or carriers [15–17]. In the course of this research, we recently chose to take advantage of the well-documented, typical biochemical phenotype of invasive solid tumors, namely the upregulation of glycolysis. Glycolysis-mediated targeting using a glucose analog as an active drug carrier is useful for the selective delivery of a drug to cancer cells, and

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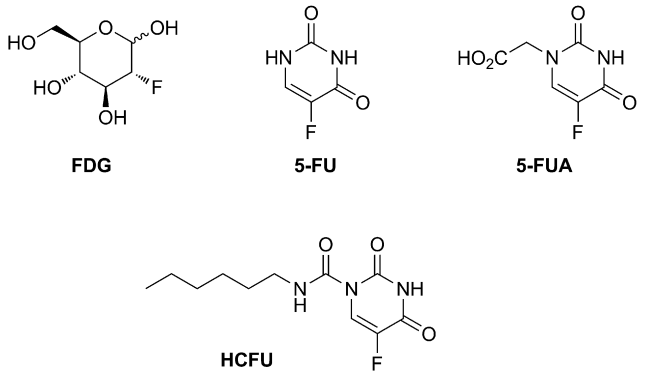


Fig. 1. Structures of FDG, 5-FU, 5-FUA and HCFU.

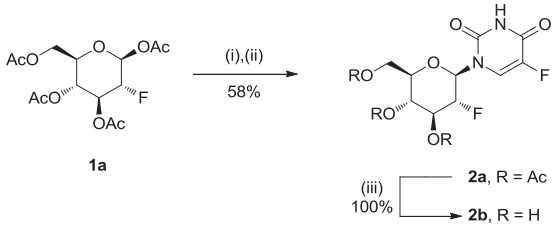
numerous studies have already been undertaken in this research area. We investigated an original tumor-targeting approach, using the glucose analog 2-fluoro-2-deoxyglucose (FDG; Fig. 1) as a low molecular weight drug carrier. This carbohydrate is known to be trapped by primary and metastatic human tumors. Thus coupling an FDG moiety with an anti-cancer agent could trap the glucoconjugate inside the tumor cells until the active moiety is released. Following this strategy, we have obtained promising results with FDG-coupled chlorambucil derivatives [18,19].

Here, we designed a series of 5-FU glycoconjugates in which the 5-FU was attached by the N1 position of the pyrimidinic ring to the C1 position of the FDG structure either by direct coupling or *via* various spacers. As the 5-FU was extensively derivatized, we took advantage of the rich related literature to adapt the stability of our 5-FU derivatives to our practical purpose. The following compounds were synthesized: direct-coupled derivatives **2a** and **2b** (Scheme 1), the 1-alkyl-5-fluorouracil derivative **3** (Scheme 2), 1-carbamoyl-5-fluorouracil derivatives **6a**–**c** (Scheme 3), and 1-(*N*-methylcarbamoyl)-5-fluorouracil derivatives **10b** (Scheme 5), and **19** (Scheme 6).

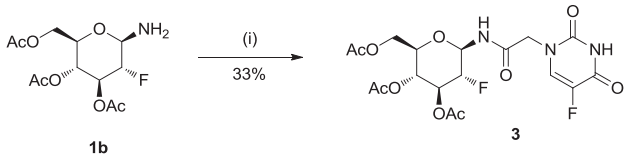
Indeed, galactopyranosyl-5-fluorouracil derivatives were presented in 1999 as interesting antitumor agents having lower toxicities than the parent drug [20]. Moreover, the coupling step between the carbohydrate and the pyrimidine takes place *via* a simple and well described chemical reaction. This led us to first investigate the direct coupling between FDG and 5-FU with the synthesis of derivatives **2a** and **2b**.

Also, we synthesized the 1-alkyl-5-fluorouracil derivative **3**. According to the literature [21], this derivative should have anti-carcinogenic activity and allow a slow 5-FU release.

Finally, carbamoyl derivatives represent a deeply investigated class of compounds, which is due to their suitable properties for oral administration, as referred to before. This can be illustrated by the clinical development of 1-hexylcarbamoyl-5-fluorouracil (HCFU; Fig. 1), which was discovered in Japan in 1977. Since then, several reports were published with regards to their physicochemical and biological properties [22–27]. Interestingly, binding affinities to



Scheme 1. Synthesis of **2a**–**b**. Reagents and conditions: (i) 5-FU, HMDS, TCS, CH_3CN; (ii) $SnCl_4$; (iii) MeONa/MeOH.



Scheme 2. Synthesis of **3**. Reagents and conditions: (i) 5-FUA, DCC, HOBt, DMF.

plasmatic proteins result in higher stability and resistance to hydrolysis in biological media. Thus, Buur and Bundgaard [25] reported that if arylcarbamoyl-5-fluorouracil derivatives display very short biological half-lives, alkylcarbamoyl-5-fluorouracil have biological half-live that might perfectly meet our requirements in terms of stability for potential 5-FU prodrugs. Moreover, their stability can be increased by varying the length of the alkyl chain. Therefore, we synthesized compounds **6a**, **6b** and **6c**, which display alkyl chains with one, three and five methylene groups, respectively. Finally, as a consequence of a more hindered environment of the carbamoyl linkage, 1-(*N*-methylcarbamoyl)-5-fluorouracil derivatives have been reported to have higher stability than their non-methylated analogs [25]. This led us to design the two last compounds of our set: derivatives **10b** and **19**, which present an alkyl and an aryl spacer arm respectively. We tried to figure out how the length of the aliphatic spacer arm would affect the stability of our derivatives. Thus, two synthetic pathways leading to **10a**, which only differs from **10b** by the size of the spacer, were investigated. Despite our efforts, this compound was not isolated but we chose to report the synthesis of the intermediates.

Synthesis and full characterization of 5-FU derivatives are described. Additionally, a new HPLC method to investigate both their plasma and chemical stability was developed. The method was used for the determination of the *in vitro* release profiles of 5-FU from starting derivatives in three different media: Phosphate Buffer Saline (PBS), human plasma and cell culture medium (MEM), under physiological pH and temperature conditions, which gave access to half-lives of the designed compounds. Finally their anti-proliferative activity was assessed *in vitro* by a fluorometric assay against two human solid cancer and one healthy cell lines.

2. Results and discussion

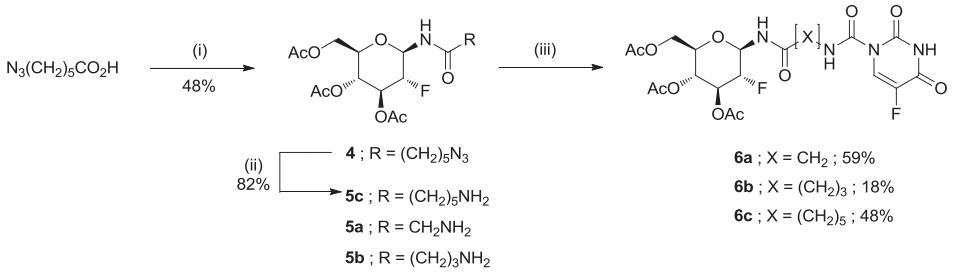
2.1. Chemistry

The synthesis of all the 5-FU derivatives was designed as follows: first, functionalized FDG analogs **1a** and **1b**, which were key intermediates, were synthesized as reported previously [18]. On the anomeric position of these precursors was grafted the spacer arm, which was most often built in several steps. In a final coupling step, the desired linking bond between 5-FU (N1 position) or (5-fluorouracil-1-yl) acetic acid (5-FUA; Fig. 1), and the sugar moiety was formed.

2.1.1. Synthesis of *2a* and *2b* (Scheme 1)

We synthesized the glyconjugate **2a** and its deacetylated analog **2b**, for which the 5-FU was directly linked to the FDG moiety.

Compound **2a** was prepared using a previously reported method for nucleoside synthesis [28], starting from 5-FU and peracetylated FDG **1a** [18]. First, 5-FU reacts with hexamethyldisilazane (HMDS) and trimethylchlorosilane (TCS) in dry acetonitrile under argon to form the intermediate 2,4-bis(trimethylsilyl)-5-fluorouracil. This species then reacts with **1a** in the presence of Lewis acid $SnCl_4$ to form **2a** as the β form only. The stereochemistry was confirmed from ^{1}H NMR data: $J_{1,2} = 9$ Hz $= J_{ax,ax}$ [18]. Subsequent Zemplèn



Scheme 3. Synthesis of **6a**–**c**. Reagents and conditions: (i) **1b**, DCC, HOBt, DMF; (ii) H_2 Pd/C, DCM/MeOH; (iii) 5-FU, triphosgene, pyridine.

deacetylation [29,30] of **2a** using sodium methylate led to **2b** in quantitative yield.

*2.1.2. Synthesis of 1-alkyl-5-fluorouracil derivative **3** (Scheme 2)*

5-FUA which was obtained in two steps, using a previously described general method [31], was a key intermediate for the synthesis of the 1-alkyl-5-fluorouracil derivative **3**. The reaction of glucosamine **1b** with 5-FUA, activated by the coupling agents *N*,*N*′-dicyclohexylcarbodiimide (DCC) and hydroxybenzotriazole (HOBt), led to the desired compound **3**.

*2.1.3. Synthesis of 1-carbamoyl-5-fluorouracil derivatives **6a**–**c** and intermediates **4**, **5a**–**c** (Scheme 3)*

To obtain the derivatives **6a**–**c** and form the carbamoyl bond, we used one out of the three main synthetic methods described for the synthesis of 1-carbamoyl-5-fluorouracil derivatives [22,24]. However, we preferred to replace phosgene by its solid synthetic equivalent triphosgene, which is safer to handle [32].

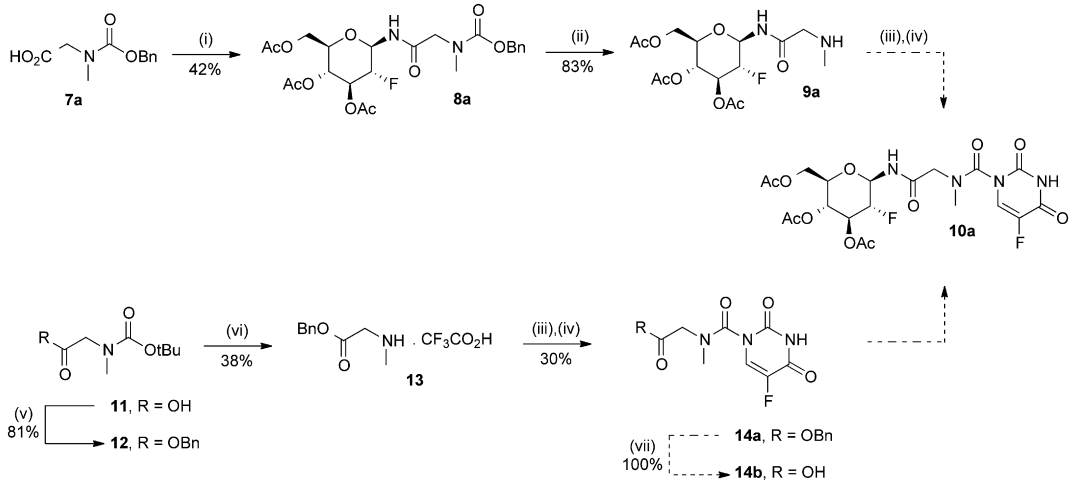
The carbamoyl bond formation being the final step of the syntheses, primary amines **5a**–**c** had to be obtained first. Syntheses of amines **5a** and **5b** were previously described by our team [18], and amine **5c** was obtained in two steps, starting from 6-azidohexanoic acid (**4**), which was prepared according to a previously described method [33]: the reaction of glucosamine **1b** with 6-azidohexanoic acid, activated by the coupling agents DCC and HOBt, gave the azido compound **4**. The subsequent hydrogenolysis of the azide function was carried out with palladium on charcoal (Pd/C) and gave the desired product **5c** in good yield (82%).

To form the carbamoyl bond, 5-FU was condensed with triphosgene in a basic medium at 0 °C to form the unstable 1-chloroformyl-5-fluorouracil species first, which then reacted *in situ* with the appropriate aliphatic primary amine **5a**–**c** to give the desired compound **6a**–**c**, respectively. For this final step, the yields ranged from 18 to 60%. The formation of the carbamoyl bond was confirmed by ^{13}C NMR, which showed the appearance of a new signal at 145.6–150.8 ppm (NHCON).

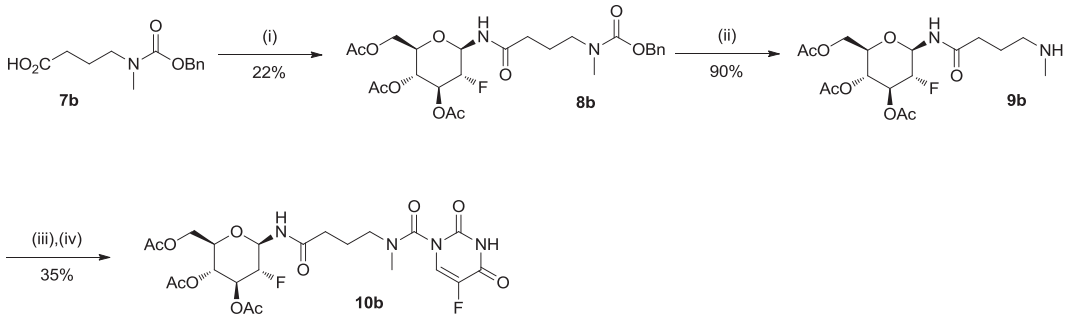
2.1.4. Synthesis of 1-(N-methylcarbamoyl)-5-fluorouracil derivatives

*2.1.4.1. Derivatives **10a**–**b** and intermediates **8a**–**b**, **9a**–**b** and **12**–**14a** (Schemes 4 and 5).* Although compounds **10a** and **10b** are the *N*-methylated analogs of derivatives **6a** and **6b** respectively, their synthetic pathways were fully reconsidered.

Two synthetic routes leading to **10a** were investigated (Scheme 4). First, amine **9a** was obtained in two steps from *N*-methylated *Z*-glycine **7a** [34]. Compound **7a** was coupled with glucosamine **1b** using DCC/HOBt to give compound **8a**, which was further deprotected by hydrogenolysis to give amine **9a**. Then, a procedure adapted from Ozaki [22,24] was performed for the derivatization of 5-FU toward 1-(*N*-methylcarbamoyl)-5-fluorouracil derivatives: the suitable amine is condensed with triphosgene to give the corresponding carbamoyl chloride, which can either be isolated or used *in situ* if unstable, and then react with 5-FU to give the 1-(*N*-methylcarbamoyl)-5-fluorouracil derivatives. Unfortunately, this method was not suitable for the coupling of amine **9a** with 5-FU *via* a *N*-methylcarbamoyl bond. Therefore we investigated the second five-step synthesis depicted on Scheme 4: the salt **13** was initially obtained by esterification of acid **11** followed by selective *N*-BOC deprotection of compound **12** [34]. Then, the *N*-methylcarbamoyl bond to 5-FU was formed according to the procedure described above in a 30% yield to give intermediate **14a**. At this point, two more steps were necessary to obtain the desired



Scheme 4. Synthesis of **10a**. Reagents and conditions: (i) **1b**, DCC, HOBt, DCM; (ii) H_2 Pd/C, MeOH/THF; (iii) triphosgene, $NaHCO_3$, DCM; (iv) 5-FU, NaH, DMAc; (v) BnBr, DBU, CH_3CN; (vi) TFA, THF; (vii) H_2 Pd/C, THF.



Scheme 5. Synthesis of **10b**. Reagents and conditions: (i) $ClCO_2Et$, TEA, **1b**, DCM; (ii) H_2 Pd/C, THF/MeOH; (iii) triphosgene, $NaHCO_3$, DCM; (iv) 5-FU, NaH, DMAc.

compound **10a**: hydrolysis of benzyl ester **14a**, and subsequent coupling of acid **14b** to glucosamine **1b**. However, hydrogenolysis of the ester **14a** led to its acid analog **14b** which was very unstable and could not be isolated.

So we turned our efforts to the synthesis of a compound which presents a longer spacer arm. According to this strategy, compound **10b** was obtained in three steps, starting from derivative **7b** (Scheme 5). This latter compound was prepared from pyrrolidinone in a multi-step synthesis, according to previous reports [35–37]. First, **7b** was coupled with glucosamine **1b** by a procedure which used ethylchloroformate ($ClCO_2Et$) and triethylamine (TEA). The subsequent step, which led to secondary amine **9b**, was the removal of the benzyl carbamate *N*-protecting group by hydrogenolysis. The final derivative **10b** was obtained from secondary amine **9b** according to the *N*-methylcarbamoyl bond formation method described above, which proved to be fairly efficient in this case.

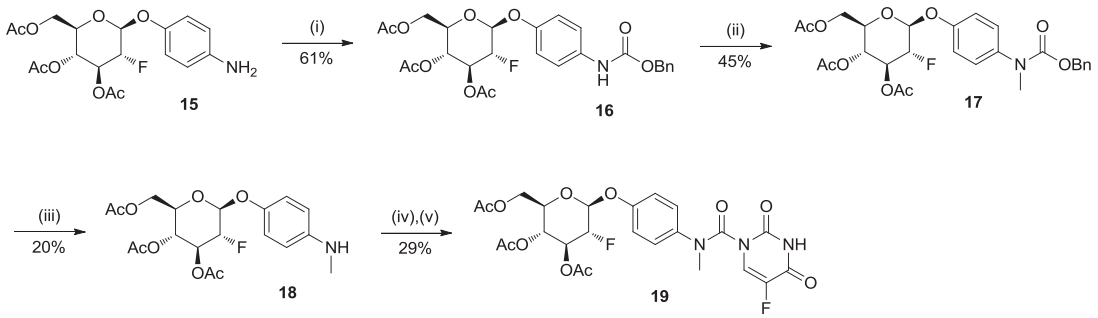
*2.1.4.2. Derivative **19** and intermediates **16**–**18** (Scheme 6).* Compound **19** was obtained in four steps starting from arylamine **15**, which was synthesized using a procedure described by our team [18]. The primary amine **15** was protected as a benzyl carbamate, allowing us to carry out the subsequent monomethylation of the compound **16** to form the derivative **17**. After hydrogenolysis of the benzyl carbamate protecting group, the secondary amine **18** was allowed to react in the final step of the synthesis to give derivative **19**.

2.2. In vitro stabilities of 5-FU derivatives

The eight final compounds **2a**, **2b**, **3**, **6a**–**c**, **10b** and **19** were incubated at 37 °C in three relevant media: Phosphate Buffer Saline (PBS), cell culture medium (MEM) and human plasma. At appropriate intervals, samples were withdrawn and prepared as described in experimental protocols. A new sensitive high-performance liquid chromatography method able to monitor both the loss of starting material and the formation of 5-FU or degraded products was developed. For all compounds, the obtained chromatograms of the stability study were compared with those of the media without 5-FU derivative. Only additional chromatographic peaks were studied.

2.2.1. Stability assessment of compounds *2a*, *3*, *10b* and *19*

Similar degradation profiles were observed for these compounds, regardless of the media. Typical HPLC-UV chromatograms obtained from their stability study at 37 °C in MEM are presented on Fig. 2. These conditions were set to analyze **5-FU**, **2a**, **3**, **10b** and **19**, which present retention times of 5.4, 18.1, 16.9, 17.6 and 19.7 min, respectively. The chromatographic profiles for these compounds evolve in the same way in the course of time: intensity of chromatographic peak of starting material decreased while two or three new peaks appeared at shorter retention times (Table 1). Nevertheless, the chromatograms obtained after 10 days showed that the 5-FU was not released in the conditions of this study. In order to characterize these degradation products (DP), the HPLC-UV method developed was combined with MS detection. The results obtained with compound **19** are presented in Fig. 3. The mass spectra in negative mode showed $[M - H]^-$ peaks at *m*/*z* 568 (**19**), *m*/*z* 484 (**DP2**) and *m*/*z* 442 (**DP3**) for the chromatographic peaks at 19.7 min, 16.2 min and 14.9 min respectively. The difference of 84 mass units between **19** and **DP2** and a difference of 42 mass units between **DP2** and **DP3** suggest a loss of two or one neutral CH_2CO. These results could be consistent with a di- and trideacetylation of compound **19**. The results obtained with **10b** are presented in Fig. 4. For each identified compound the mass spectra in positive mode showed the protonated compound $[M + H]^+$ and sodium and potassium adducts $[M + Na]^+$ and $[M + K]^+$. For the compound **10b**, the mass spectrum showed peaks at *m*/*z* 563, *m*/*z* 585 and *m*/*z* 601 for the chromatographic peak at 17.6 min. The mass spectra showed $[M + H]^+$ at *m*/*z* 521, (**DP1**), *m*/*z* 479 (**DP2**) and *m*/*z* 437 (**DP3**) for the chromatographic peaks at 16.4, 14.6 and



Scheme 6. Synthesis of **19**. Reagents and conditions: (i) $ClCO_2Bn$, $NaHCO_3$, H_2O/acetone; (ii) NaH, MeI, THF; (iii) H_2 Pd/C, THF/MeOH; (iv) triphosgene, $NaHCO_3$, DCM; (v) 5-FU, NaH, DMAc.

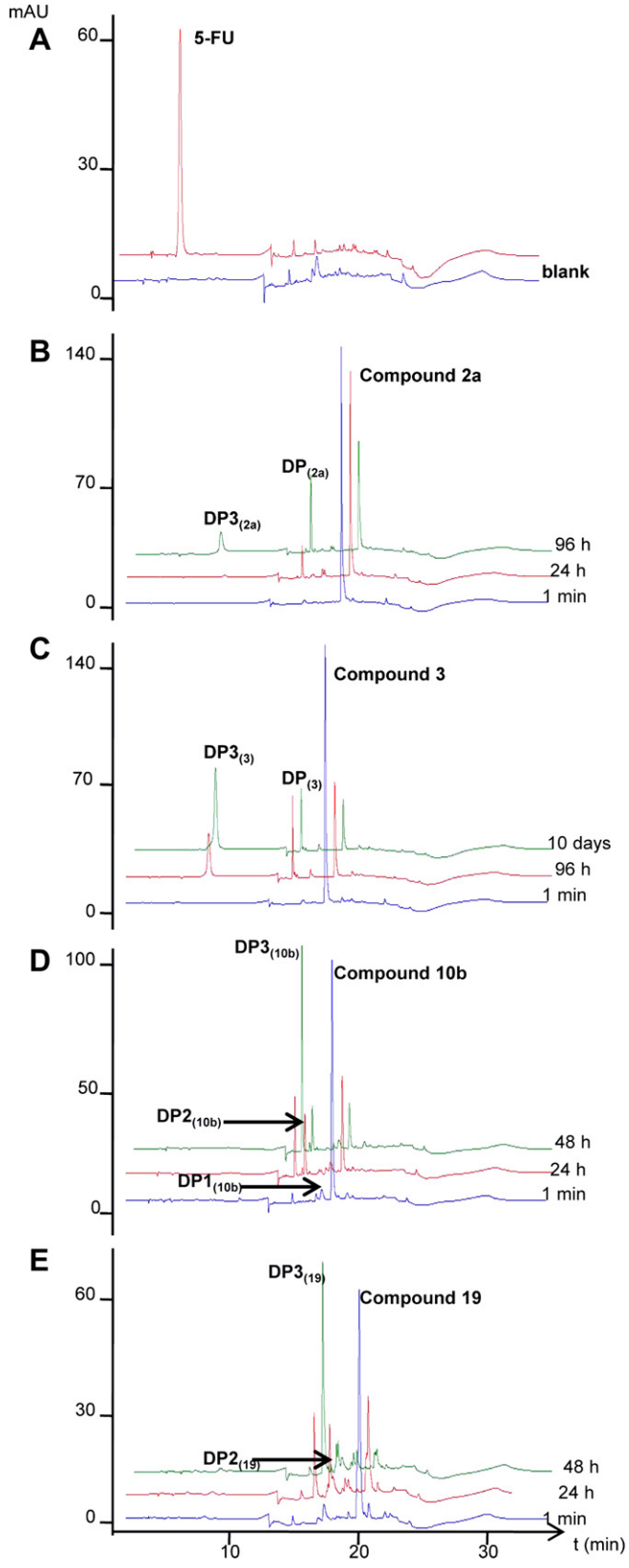


Fig. 2. (A) Typical HPLC-UV chromatograms monitored under the optimized conditions of blank (MEM) and **5-FU** at 100 μM. Typical HPLC-UV chromatograms from the stability experiments in MEM at 37 °C of compounds (B) **2a** at 1 min, 24 h and 96 h, (C) **3** at 1 min, 96 h and 10 days, (D) **10b** and (E) **19** at 1 min, 24 h and 48 h.

Table 1
Retention times of chromatographic peaks for each compound and its degraded products (DP).

Compound	Retention time (min)			
	Parent product	DP1	DP2	DP3
2a	18.1	ND	14.2[a]	7.3
3	16.9	ND	13.5[a]	6.7[a]
10b	17.6	16.4	14.6	13.4
19	19.7	ND	16.2	14.9

ND: not detected.
[a] not identified.

13.4 min respectively. The difference of 42 mass units between each compound confirmed a loss of neutral CH_2CO.

At this time, it was hypothesized that these degraded product resulted from subsequent deacetylation reactions. To have this hypothesis confirmed, the trideacetylated analog **2b** of compound **2a** was synthesized (Scheme 1) and analyzed in the same conditions. The same retention time (7.3 min) was observed for **2b** and **DP3**, which confirms that the most hydrophilic degradation product observed results from the trideacetylation of **2a**.

2.2.2. *Stability assessment of compounds **6a**, **6b** and **6c***

Similar patterns were found for all the three potential prodrugs tested in the three media. The chromatograms obtained in MEM at 37 °C for compounds **6a**–**c** are shown in Fig. 5. The retention times for **6a**, **6b** and **6c** are respectively given at 17.8 min, 17.9 min and 18.7 min. An exponential decay of 5-FU derivative concentration (Fig. 6) accompanied by only an exponential increase in 5-FU concentration was observed. The degradation profiles for compounds **6a**–**c** appeared to follow pseudo-first-order kinetics in the different media. From the individual disappearance profiles of the 5-FU derivatives, the constants rate and the half-lives were estimated from plots of log sample concentration versus time. Half-lives are reported in Table 2.

Compound **6a** was pretty unstable in PBS and MEM at physiological temperature and pH, as shown by the estimated half-lives ($t_{0.5}$: 2–3 min) obtained from the linear part of the kinetic plot. In plasma, compound **6a** also showed fast release of 5-FU under the same conditions, but less rapidly though ($t_{0.5}$ = 6 min).

For **6b**, the estimated $t_{0.5}$ values were higher in all three media ($t_{0.5}$: 6–10 min). Half-life values indicated a lower rate of hydrolysis compared to **6a**.

The qualitative patterns of **6c** hydrolysis were similar to that observed for **6b**, with the longest half-lives in the different media ($t_{0.5}$: 8–17 min).

According to these results, the release of 5-FU from each of these compounds was slower in plasma than in pH 7.4 buffer or MEM, which is very likely due to interactions with the plasmatic proteins. Moreover, an increase in the length of the linker from one (**6a**) to five carbons (**6c**) increases the half-life in the different media. For example, $t_{0.5}$ rose from 6 ± 5 min to 17 ± 3 min in plasma for compound **6c**. These data perfectly match the results previously reported in the literature [25].

3. Biological activities

A preliminary evaluation of the antiproliferative activities of all compounds was performed *in vitro* by a fluorometric assay (Resazurin reduction test (RRT)), against two human solid cancer cell lines (i.e. MCF 7: breast adenocarcinoma and PA 1: ovarian carcinoma), and a healthy cell line (i.e. a human fibroblast primary culture). Compounds **2a**, **3**, **10b** and **19** were inactive against the tested cell lines, showing IC_{50} superior to 50 μM, but compounds **6a**–**c** showed antiproliferative activities comparable to those obtained with 5-FU (Table 3). These results suggest a strong

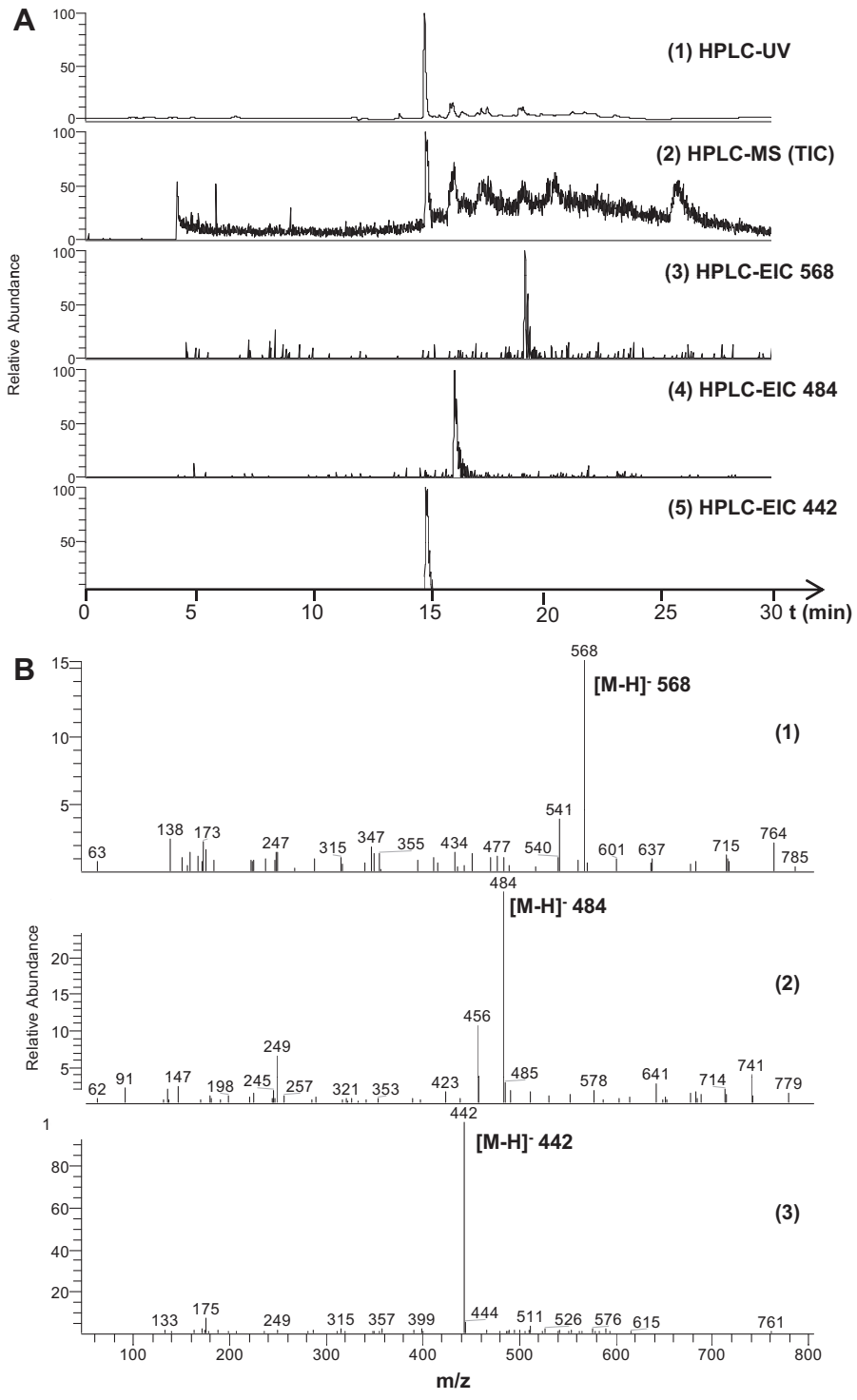


Fig. 3. Stability study of compound **19** at 37 °C in MEM. (A) HPLC-UV-MS chromatograms of **19** and its degradation products at 48 h (1) HPLC-UV chromatogram; (2) HPLC-MS total ion chromatogram (TIC) at *m*/*z* 50 to *m*/*z* 800; (3) Extracted ion current (EIC) chromatogram *m*/*z* 568 for **19** at 19.7 min; (4) EIC chromatogram *m*/*z* 484 for **DP2** at 16.2 min; (5) EIC chromatogram *m*/*z* 442 for **DP3** at 14.9 min. (B) HPLC-ESI mass spectra of **19** (1) and its degradation products **DP2** (2) and **DP3** (3).

correlation between the biological activity of our compounds, and their ability to release 5-FU efficiently.

4. Conclusions

The synthesis of eight original FDG-coupled 5-FU derivatives is reported. To adapt the stability of these derivatives to our practical purpose, we introduced various spacers and various types of chemical functions on the 5-FU structure. A new HPLC-UV method was developed to investigate the stability of the derivatives and determine their half-lives from their degradation profiles in three different media. In these *in vitro* conditions, 5-FU was not released from the direct-coupled derivative **2a**, the 1-alkyl-5-fluorouracil derivative **3** or 1-(*N*-methylcarbamoyl)-5-fluorouracil derivatives

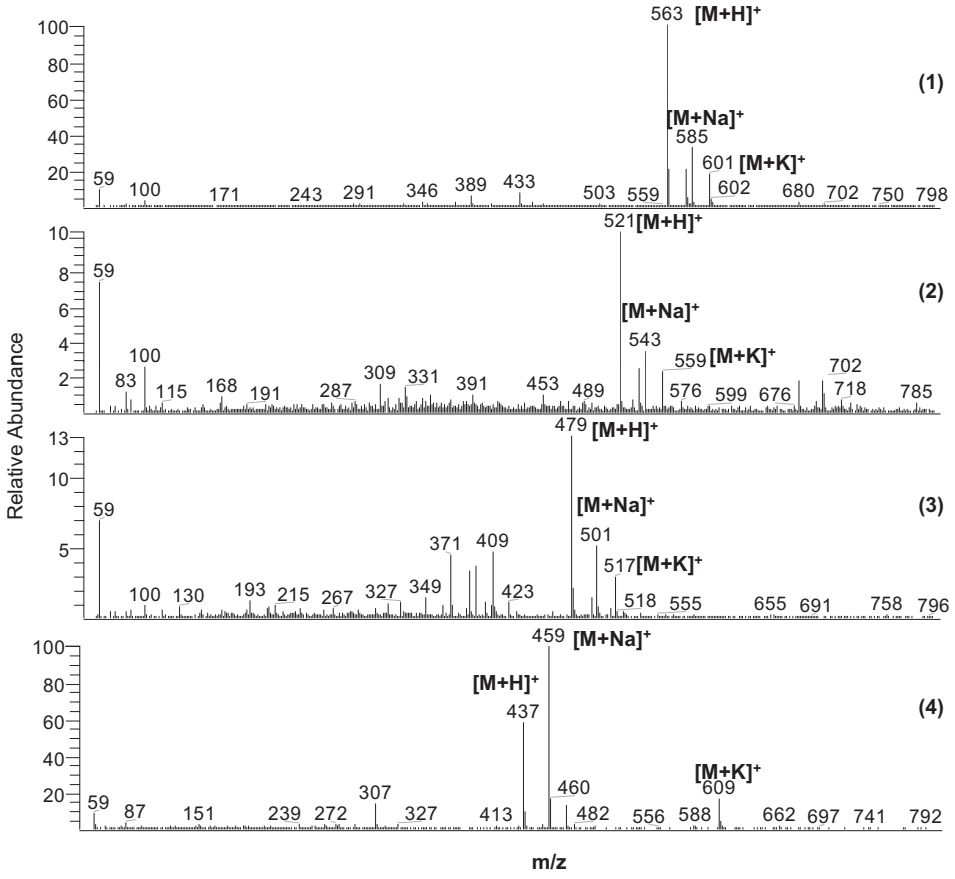


Fig. 4. Stability study of compound **10b** at 37 °C in MEM. HPLC-ESI mass spectra of **10b** (1) and its degradation products **DP1** (2), **DP2** (3) and **DP3** (4).

10b and **19**. On the other hand, 1-carbamoyl-5-fluorouracil derivatives **6a**–**c** showed 5-FU release in the three studied media, with estimated half-live values bounded by 2 and 17 min. Moreover, these compounds and 5-FU showed similar antiproliferative activities against the tested cell lines. Among the three 1-carbamoyl-5-fluorouracil derivatives, **6c** has the longest half-life, which makes it the best candidate to be further *in vivo* investigated, and potentially become an efficient 5-FU prodrug.

5. Experimental protocols

5.1. Chemistry

Chemicals were purchased from commercial sources (Sigma–Aldrich or Acros) and were used without further purification. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM-200 (4.5 T) spectrometer operating at 200 and 50 MHz respectively. Chemical shifts are reported in parts per million relative to the internal tetramethylsilane standard for ^{1}H and deuterated solvents for ^{13}C, which were purchased from C.E.A. Saclay (acetone-d_6, $\delta = 29.8$ ppm; $CDCl_3$, $\delta = 77.2$ ppm; DMSO-d_6, $\delta = 39.5$ ppm). Melting points (mp) were measured on a Stuart Scientific SMP3 melting point apparatus and were not corrected. Analytical thin-layer chromatography (TLC) was performed on SDS silica gel $60F_{254}$ precoated plates with detection by UV light and/or visualization with vanillin in sulfuric acid. Column chromatography was performed with SDS silica gel 60 (Chromagel, 35–70 μm) using the indicated solvent mixture expressed as volume/volume ratios. Mass spectra were recorded on a TSQ7000 spectrometer (Thermo Finnigan). Electrospray ionization mass spectrometry (ESI-MS) was used in positive or negative mode.

5.1.1. 1-(3,4,6-Tri-O-acetyl-2-fluoro-2-deoxy-β-D-glucopyranosyl)-5-fluorouracil, *2a*

Hexamethyldisilazane (HMDS) (0.26 mL, 1.20 mmol) and trimethylchlorosilane (TCS) (0.16 mL, 1.25 mmol) were successively added to a solution of 5-FU (150 mg, 1.15 mmol) and sugar **1a** (350 mg, 1.00 mmol) in anhydrous acetonitrile (CH_3CN) (10 mL) under argon. The mixture was stirred at room temperature (rt) for 30 min and $SnCl_4$ (0.16 mL, 1.40 mmol) was added. The solution was heated at 45 °C for 24 h and then diluted with ethyl acetate (EtOAc) (20 mL), washed twice with water (10 mL), dried over $MgSO_4$ and concentrated under vacuum. The resulting residue was purified by silica gel chromatography (cyclohexane – EtOAc, 5:5) to yield **2a** (244 mg, 0.58 mmol) as a white solid; mp 103 °C; yield 58%.

(–)-ESI-MS: 418.8 $[M - H]^-$.

^{1}H NMR ($CDCl_3$): δ 9.95 (brs, 1H, 5-FU:NH-3); 7.48 (d, 1H, $J = 5.7$ Hz, 5-FU:H-6); 6.06 (d, 1H, $J = 9.2$ Hz, H-1); 5.59 (td, 1H, $J = 13.2$ Hz, 9.2 Hz, H-3); 5.15 (t, $J = 9.2$ Hz, 1H, H-4); 4.57 (td, 1H, $J = 50.0$ Hz, 9.2 Hz, H-2); 4.28 (dd, 1H, $J = 12.6$ Hz, 4.9 Hz, H-6a); 4.18–4.03 (m, 2H, H-5, H-6b); 2.09, 2.08, 2.05 (3s, 9H, 3× CH_3).

^{13}C NMR ($CDCl_3$): δ 170.61, 169.84, 169.73 (3× $O\underline{C}OCH_3$); 156.48 (5-FU:C-4, $^{2}J_{C-F} = 26.7$ Hz); 149.16 (5-FU:C-2); 141.24 (5-FU:C-5, $^{1}J_{C-F} = 239$ Hz); 122.86 (5-FU:C-6, $^{2}J_{C-F} = 34.0$ Hz); 87.25 (C-2, $^{1}J_{C-F} = 193$ Hz); 80.15 (C-1, $^{2}J_{C-F} = 24.1$ Hz); 74.87 (C-5); 72.68 (C-3, $^{2}J_{C-F} = 19.0$ Hz); 67.49 (C-4, $^{3}J_{C-F} = 7.2$ Hz); 61.58 (C-6); 20.68, 20.58, 20.52 (3× CH_3).

5.1.2. 1-(2-Fluoro-2-deoxy-β-D-glucopyranosyl)-5-fluorouracil, *2b*

To a solution of acetylated compound **2a** (218 mg, 0.52 mmol) in dry methanol (MeOH) (5 mL) was added sodium methoxide (MeONa) (46 mg, 0.86 mmol) under argon and the mixture was stirred for 16 h at rt. After neutralization with IRC 50 Amberlite ion-

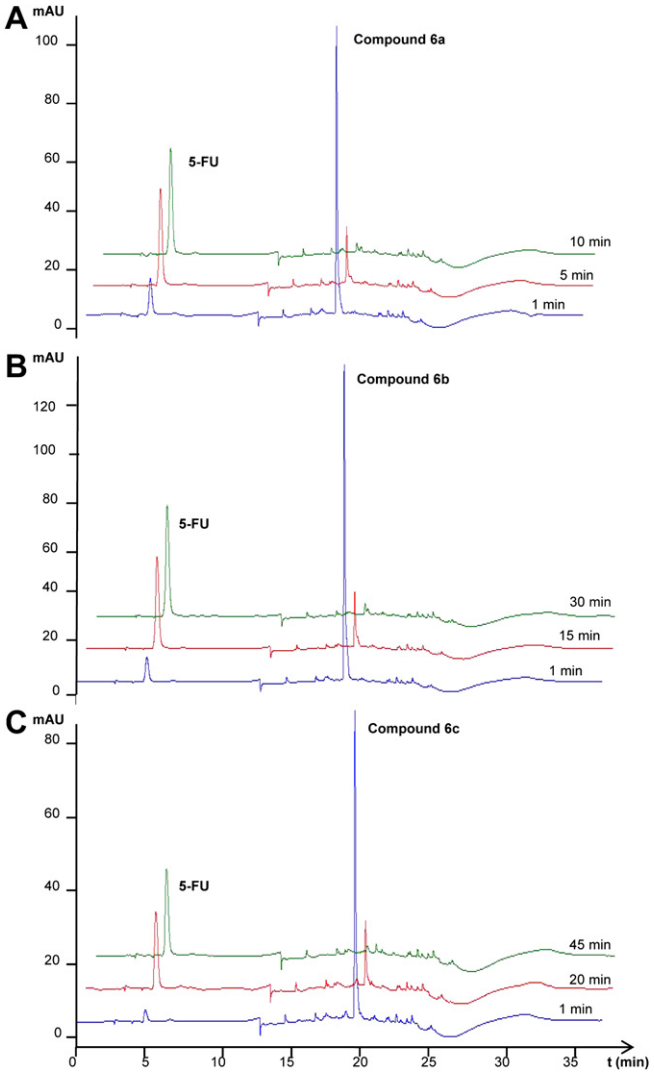


Fig. 5. Typical HPLC-UV chromatograms from the stability experiments in MEM at 37 °C of compounds (A) **6a** at 1, 5 and 10 min, (B) **6b** at 1, 15 and 30 min and (C) **6c** at 1, 20 and 45 min.

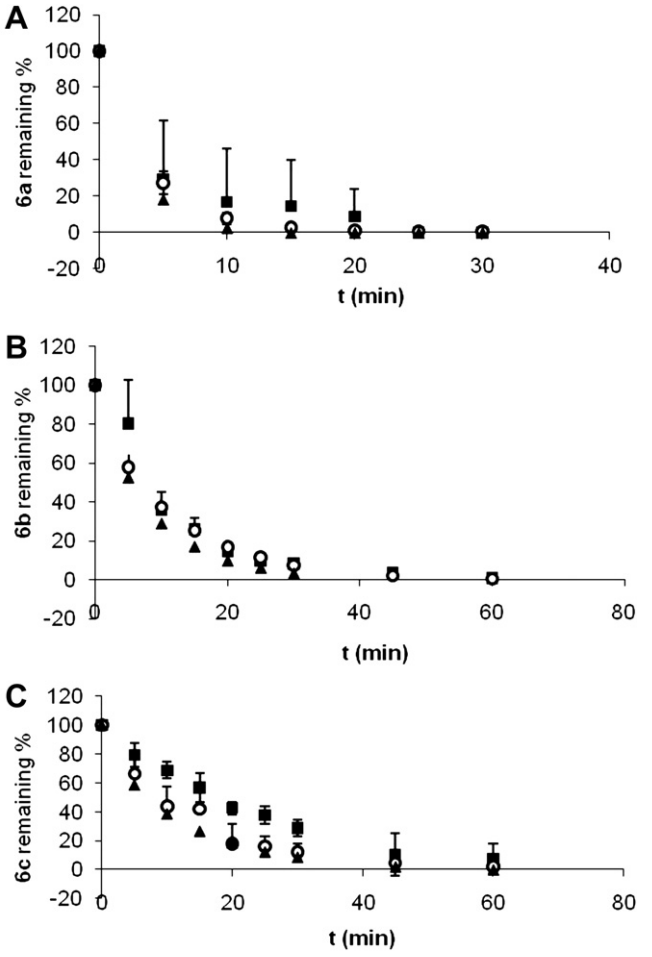


Fig. 6. Degradation profiles of compounds (A) **6a**, (B) **6b** and (C) **6c** in plasma (■), MEM (▲) and PBS (○).

exchange resin (H^+), filtration and evaporation to dryness, compound **2b** (152 mg, 0.52 mmol) was obtained as a white solid. mp 172 °C; quantitative yield.

(−)-ESI-MS: 292.7 $[M - H]^-$.

^{1}H NMR (DMSO-d_6): δ 7.98 (d, 1H, J = 7.0 Hz, 5-FU:H-6); 5.64 (d, 1H, J = 8.9 Hz, H-1); 4.48 (td, 1H, J = 51.3 Hz, 8.9 Hz, H-2); 3.68−3.20 (m, 5H, H-3, H-4, H-6a, H-6b, H-5).

^{13}C NMR (DMSO-d_6): δ 160.82 (5-FU:C-4, $^2J_{C-F}$ = 21.7 Hz); 152.64 (5-FU:C-2); 141.44 (5-FU:C-5, $^1J_{C-F}$ = 234 Hz); 124.35 (5-FU:C-6, $^2J_{C-F}$ = 34.5 Hz); 90.15 (C-2, $^1J_{C-F}$ = 183 Hz); 80.52 (C-1, $^2J_{C-F}$ = 22.4 Hz); 80.29 (C-5); 75.13 (C-3, $^2J_{C-F}$ = 15.4 Hz); 69.73 (C-4, $^3J_{C-F}$ = 7.9 Hz); 61.07 (C-6).

5.1.3. N-(3,4,6-Tri-O-acetyl-2-fluoro-2-deoxy-β-D-glucopyranosyl)-2-(5-fluorouracil-1-yl)acetamide, ***3***

Aminosugar **1b** (382 mg, 1.24 mmol) was dissolved in anhydrous dimethylformamide (DMF) (20 mL) and (5-fluorouracil-1-yl)acetic acid (5-FUA) (281 mg, 1.49 mmol), dicyclohexylcarbodiimide (DCC) (307 mg, 1.49 mmol), and hydroxybenzotriazole (HOBt) (201 mg, 1.49 mmol) were added. The solution was stirred for 48 h at rt., the dicyclohexylurea was filtered off, and the resulting solution was evaporated to dryness. The residue was dissolved twice in cold dichloromethane (DCM) (15 mL), to allow precipitation of residual dicyclohexylurea, filtered again and evaporated to dryness. The residue was purified by silica gel chromatography (DCM − MeOH, 96:4). The collected fractions were pooled, washed with a saturated $NaHCO_3$ solution and water, dried over $MgSO_4$ and evaporated under reduced pressure to give **3** (196 mg, 0.41 mmol) as a white solid; mp 129 °C; yield 33%.

(+)-ESI-MS: 478.3 $[M + H]^+$, 500.3 $[M + Na]^+$.^{1}H NMR (acetone-d_6): δ 10.51 (se, 1H, 5-FU:NH-3); 8.42 (d, 1H, J = 9.0 Hz, NHCOCH$_2$); 7.82 (d, 1H, J = 6.5 Hz, 5-FU:H-6); 5.43 (m, 2H, H-1, H-3); 4.91 (t, 1H, J = 9.7 Hz, H-4); 4.48 (s, 2H, CH_2); 4.36 (td, 1H, J = 50.6 Hz,

Table 2
Degradation half-lives of 5-FU derivatives in different media, estimated according to the first-order kinetic equation at 37 °C (Mean Values ± SEM, n = 3).

Compound	Medium	Half-life (min)
6a	PBS	3 ± 1
	MEM[a]	2
	Plasma	6 ± 5
6b	PBS	8 ± 1
	MEM[a]	6
	Plasma	10 ± 3
6c	PBS	11 ± 2
	MEM[a]	8
	Plasma	17 ± 3

[a] n = 1.

Table 3
Antiproliferative activities of 5-FU and compounds **6a**, **6b** and **6c** (IC_{50} in μM; Mean Values ± SEM, $n = 3$).

	Compounds			
	5-FU	**6a**	**6b**	**6c**
Fibroblast	11 ± 7	6.4 ± 0.7	15 ± 6	5.0 ± 0.1
MCF 7	15 ± 4	11 ± 2	23 ± 4	8.1 ± 0.8
PA 1	5 ± 1	3 ± 2	6 ± 2	4 ± 1

$J = 9.7$ Hz, H-2); 4.17 (dd, 1H, $J = 12.6$ Hz, 4.6 Hz, H-6a); 3.98 (m, 2H, H-6b, H-5); 1.98, 1.97, 1.93 (3s, 9H, 3× CH_3).

^{13}C NMR (acetone-d_6): δ 170.66, 170.14, 170.08 (3× O$\underline{C}$OCH$_3$); 168.28 (NH$\underline{C}$OCH$_2$); 158.04 (5-FU:C-4, $^2J_{C-F} = 25.9$ Hz); 150.55 (5-FU:C-2); 140.92 (5-FU:C-5, $^1J_{C-F} = 229$ Hz); 131.04 (5-FU:C-6, $^2J_{C-F} = 33.8$ Hz); 89.52 (C-2, $^1J_{C-F} = 188$ Hz); 78.16 (C-1, $^2J_{C-F} = 23.0$ Hz); 74.04 (C-5); 73.94 (C-3, $^2J_{C-F} = 19.3$ Hz); 68.85 (C-4, $^3J_{C-F} = 7.6$ Hz); 62.54 (C-6); 50.51 (CH_2); 20.58 (3× CH_3).

5.1.4. 1-{N-[2-(3,4,6-Tri-O-acetyl-2-fluoro-2-deoxy-β-D-glucopyranosyl)amino-2-oxoethyl]carbamoyl}-5-fluorouracil, **6a**

To an ice-cooled solution of 5-FU (89 mg, 0.69 mmol) in anhydrous pyridine (5 mL) was added triphosgene (204 mg, 0.69 mmol) under argon. The solution was stirred for 1 h at 0 °C, the amine **5a** (250 mg, 0.69 mmol) slowly added and the mixture was allowed to reach rt. The mixture was then stirred for 15 h and evaporated to dryness. The residue was taken up in DCM (4 mL) and the pyridine hydrochloride was filtered off. The filtrate was washed twice with a 6 N aqueous solution of hydrochloric acid (8 mL), dried over $MgSO_4$ and concentrated under vacuum. The resulting residue was purified by silica gel chromatography (cyclohexane – EtOAc, 2:8) to yield **6a** (210 mg, 0.40 mmol) as a white powder; mp 170 °C; yield 59%.

(−)ESI-MS: 518.9 $[M - H]^-$.

^{1}H NMR (DMSO-d_6): δ 12.35 (s, 1H, 5-FU:NH-3); 9.49 (t, 1H, $J = 5.4$ Hz, CH$_2$CONH); 9.05 (d, 1H, $J = 9.1$ Hz, NHCON); 8.41 (d, 1H, $J = 7.5$ Hz, 5-FU:H-6); 5.68–5.46 (m, 2H, H-1, H-3); 4.87 (t, 1H, $J = 9.2$ Hz, H-4); 4.43 (td, 1H, $J = 50.5$ Hz, $J = 9.2$ Hz, H-2); 4.24–3.93 (m, 5H, H-6a, H-6b, H-5, C$\underline{H}_2$CONH); 2.03, 2.00, 1.99 (3s, 9H, 3× CH_3).

^{13}C NMR (CF_3CO_2D): δ 170.70, 169.74, 169.35, 166.61 (CH$_2$$\underline{C}$ONH, 3× O$\underline{C}OCH_3$); 150.77 (5-FU:C-4, $^2J_{C-F} = 26.8$ Hz); 145.61, 145.01 (NHC$\underline{O}$N, 5-FU:C-2); 135.69 (5-FU:C-5, $^1J_{C-F} = 236$ Hz); 118.65 (5-FU:C-6, $^2J_{C-F} = 38.2$ Hz); 82.27 (C-2, $^1J_{C-F} = 191$ Hz); 72.40 (C-1, $^2J_{C-F} = 23.9$ Hz); 68.89 (C-3, $^2J_{C-F} = 19.8$ Hz); 68.16 (C-5); 63.14 (C-4); 57.02 (C-6); 38.53 (C$\underline{H}_2$NHCO); 13.65 (3× CH_3).

5.1.5. 1-{N-[4-(3,4,6-Tri-O-acetyl-2-fluoro-2-deoxy-β-D-glucopyranosyl)amino-4-oxobutyl]carbamoyl}-5-fluorouracil, **6b**

Compound **6b** was prepared according to the procedure described for compound **6a**, starting from 5-FU (83 mg, 0.64 mmol), in anhydrous pyridine (4 mL), triphosgene (189 mg, 0.64 mmol) and amine **5b** (250 mg, 0.64 mmol). The mixture was stirred for 17 h before work-up. Purification conditions similar to those used for compound **6a** lead to compound **6b** (86 mg, 0.16 mmol), which was obtained as a white powder; mp 90 °C; yield 18%.

(−)-ESI-MS: 546.9 $[M - H]^-$.

^{1}H NMR (acetone-d_6): δ 11.00 (brs, 1H, 5-FU:NH-3); 9.27 (brs, 1H, NH$\underline{C}$ON); 8.49 (d, 1H, $J = 7.4$ Hz, 5-FU:H-6); 8.17 (d, 1H, $J = 9.3$ Hz, C$\underline{H}_2$CONH); 5.60–5.43 (m, 2H, H-1, H-3); 5.01 (t, 1H, $J = 9.4$ Hz, H-4); 4.45 (td, 1H, $J = 50.5$ Hz, $J = 9.4$ Hz, H-2); 4.28 (dd, 1H, $J = 12.8$ Hz, 5.0 Hz, H-6a); 4.13–4.05 (m, 2H, H-5, H6b); 3.48 (q, 2H, $J = 6.8$ Hz, C$\underline{H}_2$NH); 2.40 (t, 2H, $J = 7.2$ Hz, NHCOC$\underline{H}_2$); 2.12–1.92 (m, 11H, CH$_2$C$\underline{H}_2$CH$_2$, 3× CH_3).

^{13}C NMR (acetone-d_6): δ 172.91, 170.43, 169.88 (CH$_2$$\underline{C}$ONH, 3× O$\underline{C}OCH_3$); 157.10 (5-FU:C-4, $^2J_{C-F} = 27.7$ Hz); 151.17, 150.50 (NHCON, 5-FU:C-2); 141.75 (5-FU:C-5, $^1J_{C-F} = 234$ Hz); 123.17 (5-FU:C-6, $^2J_{C-F} = 37.8$ Hz); 89.39 (C-2, $^1J_{C-F} = 187$ Hz); 77.82 (C-1, $^2J_{C-F} = 22.8$ Hz); 73.88 (C-3, $^2J_{C-F} = 18.6$ Hz); 73.70 (C_5); 68.80 (C-4, $^3J_{C-F} = 7.3$ Hz); 62.41 (C-6); 40.89 (C$\underline{H}_2$NHCO); 33.43 (C$\underline{H}_2$CONH); 28.46 (CH$_2$C$\underline{H}_2$CH$_2$); 20.37 (3× CH_3).

5.1.6. 6-Azido-N-(3,4,6-tri-O-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosyl)hexanamide, **4**

Compound **4** was prepared according to the procedure described for compound **3**, starting from 6-azidohexanoic acid (1.00 g, 6.36 mmol), DCC (1.57 g, 7.63 mmol), HOBt (1.03 g, 7.63 mmol), and aminosugar **1b** (2.34 g, 4.24 mmol) in DMF (45 mL). The mixture was stirred for 18 h before work up. Compound **4** (913 mg, 2.05 mmol) was obtained as a white powder. mp 84 °C; yield 48%.

(−)-ESI-MS: 444.9 $[M - H]^-$.

^{1}H NMR ($CDCl_3$): δ 6.99 (d, 1H, $J = 9.2$ Hz, NH); 5.46–5.31 (m, 2H, H-1, H-3); 5.01 (t, 1H, $J = 9.5$ Hz, H-4); 4.39 (td, 1H, $J = 50.2$ Hz, 9.5 Hz, H-2); 4.37 (dd, 1H, $J = 12.6$ Hz, 4.2 Hz, H-6a); 4.05 (dd, 1H, $J = 12.6$ Hz, 1.8 Hz, H-6b); 3.88 (ddd, 1H, $J = 12.6$ Hz, 4.2 Hz, 1.8 Hz, H-5); 3.28 (t, 2H, $J = 7.6$ Hz, CH$_2$N$_3$); 2.29 (t, 2H, $J = 7.2$ Hz, C$\underline{H}_2$CONH); 2.08, 2.06, 2.05 (3s, 9H, 3× CH_3); 1.73–1.62 (m, 4H, C$\underline{H}_2$C$\underline{H}_2$CH$_2$CH$_2$N$_3$); 1.59–1.27 (m, 2H, C$\underline{H}_2$CH$_2$N$_3$).

IR (KBr) cm^{-1}: ν 3342, 2099, 1746, 1681, 1228, 1034.

5.1.7. 3-Amino-N-(3,4,6-tri-O-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosyl)hexanamide, **5c**

To a suspension of 10% Pd/C (130 mg) in DCM/MeOH (60/30 mL) was added the azido compound **4** (657 mg, 1.47 mmol). The mixture was hydrogenated at atmospheric pressure for 20 h, filtered through celite, and concentrated to yield a residue, which was taken up in diisopropyl ether (10 mL). Filtration of the precipitate gave the amine **5c** (505 mg, 1.20 mmol) as a yellow hygroscopic powder; mp 163 °C; yield 82%.

(+)-ESI-MS: 421.0 $[M + H]^+$.

^{1}H NMR ($CDCl_3$): δ 7.98 (d, 1H, $J = 8.9$ Hz, NH); 5.42–5.29 (m, 2H, H-1, H-3); 5.01 (t, 1H, $J = 9.8$ Hz, H-4); 4.52 (td, 1H, $J = 49.9$ Hz, 9.8 Hz, H-2); 4.30 (dd, 1H, $J = 12.9$ Hz, 4.8 Hz, H-6a); 4.02 (d, 1H, $J = 12.9$ Hz, H-6b); 3.84 (m, 1H, H-5); 2.94 (m, 2H, C$\underline{H}_2$NH$_2$); 2.30 (m, 2H, C$\underline{H}_2$CONH); 2.06, 2.05, 2.02 (3s, 9H, 3× CH_3); 1.87–1.23 (3m, 8H, C$\underline{H}_2$C$\underline{H}_2$C$\underline{H}_2$CH$_2$N$\underline{H}_2$).

5.1.8. 1-{N-[6-(3,4,6-Tri-O-acetyl-2-fluoro-2-deoxy-β-D-glucopyranosyl)amino-6-oxohexyl]carbamoyl}-5-fluorouracil, **6c**

Compound **6c** was prepared according to the procedure described for compound **6a**, starting from 5-FU (51 mg, 0.39 mmol), in anhydrous pyridine (3 mL), triphosgene (115 mg, 0.39 mmol) and amine **5c** (164 mg, 0.39 mmol). The mixture was stirred for 15 h before work-up. Purification conditions similar to those used for compound **6a** lead to compound **6c** (109 mg, 0.19 mmol), which was obtained as a white powder; mp 134 °C; yield 48%.

(−)-ESI-MS: 575.1 $[M - H]^-$.

^{1}H NMR (DMSO-d_6): δ 12.27 (brs, 1H, 5-FU:NH-3); 9.12 (t, 1H, $J = 5.6$ Hz, NH$\underline{C}$ON); 8.78 (d, 1H, $J = 8.9$ Hz, CH$_2$CONH); 8.38 (d, 1H, $J = 7.5$ Hz, 5-FU:H-6); 5.75–5.39 (m, 2H, H-1, H-3); 4.85 (t, 1H, $J = 9.0$ Hz, H-4); 4.37 (td, 1H, $J = 50.3$ Hz, $J = 9.0$ Hz, H-2); 4.19–3.92 (m, 3H, H-5, H-6a, H-6b); 3.27 (m, 2H, C$\underline{H}_2$NH); 2.19–1.98 (m, 11H, CH_2CO, 3× CH_3); 1.52 (m, 4H, 2× CH_2); 1.27 (m, 2H, CH_2).

^{13}C NMR (DMSO-d_6): δ 173.40, 170.57, 170.11, 169.93 (CH$_2$$\underline{C}$ONH, 3× O$\underline{C}OCH_3$); 157.58 (5-FU:C-4, $^2J_{C-F} = 26.9$ Hz); 150.79, 150.06 (NHCON, 5-FU:C-2); 141.21 (5-FU:C-5, $^1J_{C-F} = 232$ Hz); 123.43 (5-FU:C-6, $^2J_{C-F} = 37.3$ Hz); 89.04 (C-2, $^1J_{C-F} = 186$ Hz); 77.00 (C-1, $^2J_{C-F} = 22.5$ Hz); 73.34 (C-3, $^2J_{C-F} = 19.3$ Hz); 72.46 (C-5); 68.32 (C-4); 62.18 (C-6); 35.87 (C$\underline{H}_2$CONH); 29.05, 26.34, 25.00 (C$\underline{H}_2$C$\underline{H}_2$C$\underline{H}_2$C$\underline{H}_2$NHCO); 21.02 (3× CH_3). *Signal of* C$\underline{H}_2$NHCO *in the solvent signal.*

5.1.9. N-(3,4,6-Tri-O-acetyl-2-fluoro-2-deoxy-β-D-glucopyranosyl)-2-(N′-benzyloxycarbonyl-N′-methyl)aminoethanamide, ***8a***

Compound **8a** was prepared according to the procedure described for compound **3**, starting from aminosugar **1b** (826 mg, 2.69 mmol), DCC (666 mg, 3.23 mmol) and HOBt (436 mg, 3.23 mmol) in anhydrous DCM (10 mL). The mixture was stirred for 24 h before work-up. The crude product was purified by silica gel chromatography (cyclohexane – EtOAc, 4:6) to give compound **8a** (580 mg, 1.13 mmol) as a white powder; mp 116 °C; yield 42%.

(−)-ESI-MS: 511.07 $[M - H]^-$.

^{1}H NMR ($CDCl_3$): δ 7.34 (m, 5H, $H_{arom.}$); 7.12 (d, 1H, J = 8.9 Hz, NH); 5.44–5.28 (m, 2H, H-1, H-3); 5.15 (s, 2H, $NCOOCH_2$); 5.02 (t, 1H, J = 9.4 Hz, H-4); 4.29 (dd, 1H, J = 12.6 Hz, 4.4 Hz, H-6a); 4.27 (td, 1H, J = 50.5 Hz, 9.4 Hz, H-2); 4.07–4.01 (m, 3H, CH_2CONH, H-6b); 3.88 (dd, 1H, J = 12.6 Hz, 4.4 Hz, H-5); 3.00 (s, 3H, NCH_3); 2.08 (s, 3H, CH_3); 2.04 (s, 6H, 2× CH_3).

5.1.10. N-(3,4,6-Tri-O-acetyl-2-fluoro-2-deoxy-β-D-glucopyranosyl)-4-methylaminoethanamide, ***9a***

N-Benzyloxycarbonyl (*N*-Cbz) protected derivative **8a** (537 mg, 1.05 mmol) was added, with stirring, to a suspension of 10% Pd/C (145 mg) in THF/MeOH (5/5 mL). The mixture was stirred at rt for 5 h, filtered through celite, and evaporated under reduced pressure to give the expected compound **9a** (330 mg, 0.87 mmol) as a gray solid which was used without further purification; yield 83%.

(−)-ESI-MS: 378.97 $[M - H]^-$.

^{1}H NMR ($CDCl_3$): δ 8.08 (d, 1H, J = 9.8 Hz, NHCO); 5.46–5.30 (m, 2H, H-1, H-3); 5.07 (t, 1H, J = 9.6 Hz, H-4); 4.36 (td, 1H, J = 50.6 Hz, 9.6 Hz, H-2); 4.30 (dd, 1H, J = 12.4 Hz, 4.5 Hz, H-6a); 4.08 (dd, 1H, J = 12.4 Hz, 2.1 Hz, H-6b); 3.86 (ddd, 1H, J = 12.4 Hz, 4.5 Hz, 2.1 Hz, H-5); 3.31 (d, 2H, J = 4.4 Hz, CH_2); 2.44 (s, 3H, $NHCH_3$); 2.08, 2.07, 2.04 (3s, 9H, 3× CH_3).

5.1.11. Benzyl 2-((tert-butoxycarbonyl)methylamino)acetate, ***12***

To a solution of **11** (1.44 g, 7.6 mmol) in CH_3CN were added 1,8-diaza-bicyclo[5.4.0]undec-7-ene (DBU) (1.1 mL, 7.6 mmol) and benzyl bromide (BnBr) (0.9 mL, 7.30 mmol). After 5 h at 60 °C, the reaction mixture was allowed to cool to rt and evaporated to dryness. The residue was diluted with EtOAc (60 mL) and washed successively with a saturated solution of sodium bicarbonate ($NaHCO_3$) (50 mL), an aqueous potassium hydrogen sulfate solution (1 N) (50 mL), and brine (40 mL). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to give compound **12** (1.73 g, 6.2 mmol) as a yellow oil; yield 81%.

^{1}H NMR ($CDCl_3$): δ 7.34 (m, 5H, $H_{arom.}$); 5.17, 5.15 (2s, 2H, $COOCH_2$); 4.01, 3.92 (2s, 2H, CH_2N); 2.92, 2.91 (2s, 3H, NCH_3); 1.46, 1.42, 1.37 (3s, 9H, 3× CH_3).

5.1.12. Benzyl 2-(methylamino)acetate, trifluoroacetic acid salt, ***13***

To a solution of **12** (2.00 g, 7.16 mmol) in THF (8 mL) was added trifluoroacetic acid (TFA) (8 mL). The reaction mixture was stirred at rt for 20 min, and evaporated to dryness to give compound **13** (755 mg, 2.72 mmol) as a yellow oil, which was used without further purification; yield 38%.

^{1}H NMR ($CDCl_3$): δ 7.36 (s, 5H, $H_{arom.}$); 5.17 (s, 2H, $COOCH_2$); 3.41 (s, 2H, CH_2N); 2.44 (s, 3H, CH_3N).

5.1.13. 1-[N-(2-Benzyloxy-2-oxoethyl)-N-methylcarbamoyl]-5-fluorouracil, ***14a***

A solution of salt **13** (150 mg, 0.84 mmol) in DCM (1.5 mL) is added dropwise to a slurry of $NaHCO_3$ (141 mg, 1.68 mmol) and triphosgene (164 mg, 0.55 mmol) in dry DCM (2 mL) at 10–15 °C over 10 min under argon. The reaction mixture was stirred at rt for 1 h. The reaction mass was then filtered off to remove NaCl and the filtrate was concentrated under vacuum to give the carbamoyl chloride of amine **13** (200 mg, 0.83 mmol) which was used without further purification.

To a solution of 5-FU (108 mg, 0.83 mmol) in freshly distilled *N*,*N*-dimethylacetamide (DMAc) (3.3 mL) was slowly added a 60% dispersion of sodium hydride (NaH) in mineral oil (32 mg, 0.83 mmol) under argon. The mixture was heated at 30 °C and the carbamoyl chloride of amine **13** in DMAc (1 mL) was added dropwise. The solution was stirred at 30 °C for 15 h before evaporation. The residue was taken up in DCM (4 mL), washed once with water, dried over $MgSO_4$ and concentrated under vacuum. The resulting residue was purified by silica gel chromatography (cyclohexane – EtOAc, 2:8) to give compound **14a** (103 mg, 0.31 mmol) as a white powder; mp 140 °C; overall yield 37%.

^{1}H NMR (DMSO-d_6): δ 11.00 (se, 1H, 5-FU:NH-3); 8.00, 7.89 (2d, 1H, J = 6.4 Hz, 5-FU:H-6); 7.38 (m, 5H, $H_{arom.}$); 5.20, 5.16 (2s, 2H, $COOCH_2$); 4.33, 4.29 (2s, 2H, CH_2N); 3.03 (s, 3H, CH_3N).

5.1.14. N-(3,4,6-Tri-O-acetyl-2-fluoro-2-deoxy-β-D-glucopyranosyl)-4-(N′-benzyloxycarbonyl-N′-methyl) aminobutanamide, ***8b***

A stirred solution of **7b** (700 mg, 2.95 mmol) in DCM (20 mL) was treated with TEA (0.50 mL, 3.54 mmol). The mixture was cooled to 0 °C, treated with $ClCO_2Et$ (0.30 mL, 3.11 mmol), and stirred at rt for 30 min. Aminosugar **1b** (824 mg, 2.68 mmol) in DCM (30 mL) was then added, and the mixture was stirred for 20 h at rt. The mixture was suspended in saturated aqueous Na_2CO_3 solution (60 mL) and extracted three times with DCM (40 mL). The organic extracts were combined, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel chromatography (cyclohexane – EtOAc, 4:6), to give *N*-Cbz protected compound **8b** (326 mg, 0.60 mmol) as a white solid. mp 62 °C; yield 22%.

(−)-ESI-MS: 539.0 $[M - H]^-$.

^{1}H NMR ($CDCl_3$): δ 8.16 (d, 1H, J = 8.7 Hz, $NHCOCH_2$); 7.36 (m, 5H, $H_{arom.}$); 5.46–5.30 (m, 2H, H-1, H-3); 5.15 (s, 2H, $NCOOCH_2$); 5.07 (t, 1H, J = 9.5 Hz, H-4); 4.44 (td, 1H, J = 50.3 Hz, 9.5 Hz, H-2); 4.30 (dd, 1H, J = 12.5 Hz, 4.4 Hz, H-6a); 4.08 (dd, 1H, J = 12.5 Hz, 1.7 Hz, H-6b); 3.85 (m, 1H, J = 12.5 Hz, 4.4 Hz, 1.7 Hz, H-5); 3.58–3.16 (m, 2H, CH_2N); 2.91 (s, 3H, NCH_3); 2.20 (m, 2H, CH_2CONH); 2.08, 2.06, 2.04 (3s, 9H, 3× CH_3); 1.87 (m, 2H, $CH_2CH_2CH_2$).

5.1.15. N-(3,4,6-Tri-O-acetyl-2-fluoro-2-deoxy-β-D-glucopyranosyl)-4-methylaminobutanamide, ***9b***

Compound **9b** was prepared according to the procedure described for compound **9a**, starting from compound **8b** (295 mg, 0.55 mmol) and 10% Pd/C (60 mg) in THF/MeOH (20/20 mL). The mixture was stirred for 30 min before work-up. The crude product was purified by silica gel chromatography (DCM – MeOH, 9:1) to give compound **9b** (200 mg, 0.49 mmol) as a yellow gum; yield 90%.

(+)ESI-MS: 406.0 $[M + H]^+$.

^{1}H NMR ($CDCl_3$): δ 5.45–5.29 (m, 2H, H-1, H-3); 5.04 (t, 1H, J = 9.6 Hz, H-4); 4.34 (td, 1H, J = 50.5 Hz, 9.6 Hz, H-2); 4.31 (dd, 1H, J = 12.5 Hz, 4.2 Hz, H-6a); 4.07 (dd, 1H, J = 12.5 Hz, 1.8 Hz, H-6b); 3.86 (m, 1H, H-5); 2.69 (t, 2H, J = 6.2 Hz, CH_2NH); 2.43–2.38 (m, 5H, CH_3, CH_2CONH); 2.08, 2.07, 2.04 (3s, 9H, 3× CH_3); 1.85 (m, 2H, $CH_2CH_2CH_2$).

5.1.16. 1-{N-[4-(3,4,6-Tri-O-acetyl-2-fluoro-2-deoxy-β-D-glucopyranosyl)amino-4-oxobutyl]-N′-methylcarbamoyl}-5-fluorouracil, ***10b***

Compound **10b** was prepared in two steps according to the procedure described for compound **14a**.

First, the carbamoyl chloride was formed starting from $NaHCO_3$ (84 mg, 1.00 mmol), triphosgene (98 mg, 0.33 mmol) in dry DCM (4 mL) and a solution of amine **9b** (204 mg, 0.50 mmol) in DCM (1.5 mL).

The reaction mixture was stirred at rt for 20 h before work-up. The resulting carbamoyl chloride of amine **9b** (145 mg, 0.32 mmol) was a light yellow oil, used without further purification.

To a solution of 5-FU (40 mg, 0.31 mmol) in DMAc (2 mL) was slowly added a 60% dispersion of NaH in mineral oil (12 mg, 0.31 mmol) under argon. The mixture was heated at 45 °C and the carbamoyl chloride of amine **9b** (145 mg, 0.31 mmol) in DMAc (1 mL) was added dropwise. The solution was stirred at 45 °C for 8 h and left to stand 12 h at rt before evaporation. The residue was taken up in DCM (4 mL), washed once with water (6 mL), dried over $MgSO_4$ and concentrated under vacuum. The resulting residue was purified by silica gel chromatography (DCM – MeOH, 95:5) to yield **10b** (95 mg, 0.17 mmol) as a white solid; mp 81 °C; overall yield 35%.

(+)ESI-MS: 563.2 $[M + H]^+$.

^{1}H NMR (DMSO-d_6): δ 8.86, 8.83 (2d, 1H, J = 8.9 Hz, NHCO); 8.13, 8.08 (2d, 1H, J = 6.3 Hz, 5-FU:H-6); 5.60–5.42 (m, 2H, H-1, H-3); 4.85 (t, 1H, J = 9.7 Hz, H-4); 4.38 (td, 1H, J = 50.5 Hz, 9.7 Hz, H-2); 4.18–3.92 (m, 3H, H-6a, H-6b, H-5); 2.96, 2.90 (2s, 3H, NCH_3); 2.25–2.13 (m, 2H, CH_2CONH); 2.08, 2.03, 1.98 (3s, 9H, 3× CH_3); 1.96–1.77 (m, 2H, $CH_2CH_2CH_2$); *signal of CH_2N in H_2O signal.*

^{13}C NMR ($CDCl_3$): δ 172.97 ($NHCOCH_2$); 170.75, 170.07, 169.82 (3× $OCOCH_3$); 156.99 (5-FU:C-4, $^2J_{C-F}$ = 26.5 Hz); 150.81 (5-FU:C-2); 148.19 (NCON); 141.11 (5-FU:C-5, $^1J_{C-F}$ = 222 Hz); 125.53 (5-FU:C-6, $^2J_{C-F}$ = 34.4 Hz); 88.27 (C-2, $^1J_{C-F}$ = 189 Hz); 77.16 (C-1, $^2J_{C-F}$ = 24.1 Hz); 73.72 (C-5); 73.61 (C-3, $^2J_{C-F}$ = 19.2 Hz); 68.14 (C-4, $^3J_{C-F}$ = 7.3 Hz); 61.91 (C-6); 49.65 (CH_2NCO); 37.52 (NCH_3); 32.38 ($NHCOCH_2$); 21.79 ($CH_2CH_2CH_2$); 20.82, 20.78, 20.71 (3× $OCOCH_3$).

5.1.17. [4-(3,4,6-Tri-O-acetyl-2-fluoro-2-deoxy-β-D-glucopyranosyloxy)phenyl]benzyl carbamate, **16**

To a solution of **15** (713 mg, 1.79 mmol) in a mixture of water (3 mL) and acetone (6 mL) was added $NaHCO_3$ (316 mg, 3.76 mmol). The solution was cooled to 0 °C, treated with benzyl chloroformate ($ClCO_2Bn$) (1.84 mmol, 0.26 mL) dropwise, and stirred at rt for 14 h. The reaction mixture was then poured onto ice (50 g) and the precipitate formed was filtered off, washed with water (50 mL) and dried. The resulting solid was purified by silica gel chromatography (cyclohexane – EtOAc, 6:4) to give an oil, which was taken up in diethyl ether. Filtration of the precipitate gave the amine **16** (586 mg, 1.10 mmol) as a white fluffy powder. mp 131 °C; yield 61%.

(−)-ESI-MS: 531.0 $[M - H]^-$.

^{1}H NMR ($CDCl_3$): δ 7.38 (m, 5H, $H_{arom.\ Bn}$); ν_A = 7.32, ν_B = 7.02 (AA′BB′, J_{AB} = 8.9 Hz, $4H_{arom.}$); 6.68 (brs, 1H, NH); 5.39 (td, 1H, J = 14.4 Hz, 9.2 Hz, H-3); 5.19 (s, 2H, $NHCOOCH_2$); 5.14–5.03 (m, 2H, H-1, H-4); 4.55 (td, 1H, J = 50.3 Hz, 9.2 Hz, H-2); 4.29 (dd, 1H, J = 12.3 Hz, 5.2 Hz, H-6a); 4.15 (dd, 1H, J = 12.3 Hz, 2.1 Hz, H-6b); 3.84 (m, 1H, H-5); 2.11, 2.08, 2.05 (3s, 9H, 3× CH_3).

5.1.18. N-[4-(3,4,6-Tri-O-acetyl-2-fluoro-2-deoxy-β-D-glucopyranosyloxy)phenyl]-N-methyl-benzylcarbamate, **17**

Compound **16** (570 mg, 1.07 mmol) and methyl iodide (MeI) (67 μL, 1.07 mmol) were dissolved in THF (8 mL) and the solution was cooled to 0 °C in a flask protected from moisture. A 60% NaH dispersion in mineral oil (60 mg, 2.50 mmol) was then added portionwise and cautiously with gentle stirring. The suspension was stirred at rt for 5 h. A slight excess of MeI was added to complete the reaction. EtOAc was then added (4 mL), followed by water (4 mL), dropwise, to destroy the excess of NaH. The solution was evaporated to dryness, and the oily residue partitioned between ether (40 mL) and water (40 mL). The ether layer was washed with aqueous saturated $NaHCO_3$ solution (30 mL), and the combined aqueous extracts acidified to pH 2 with 5 N aqueous HCl solution. The product was extracted into EtOAc (50 mL). The combined extracts were washed with water (35 mL), 5% aqueous sodium thiosulphate (30 mL), and again water (30 mL), dried over $MgSO_4$, filtered and evaporated to dryness. The resulting residue was purified by chromatography on alumina (cyclohexane – EtOAc, 6:4) to yield **17** (263 mg, 0.48 mmol) as a white gum; yield 45%.

^{1}H NMR ($CDCl_3$): δ 7.33 (m, 5H, $H_{arom.\ Bn}$); ν_A = 7.20, ν_B = 7.05 (AA′BB′, J_{AB} = 8.8 Hz, $4H_{arom.}$); 7.05 (d, 2H, J = 8.7 Hz, $H_{arom.}$); 5.44 (td, 1H, J = 14.6 Hz, 9.2 Hz, H-3); 5.17–5,02 (m, 4H, H-1, H-4, $COOCH_2$); 4,59 (td, 1H, J = 50.4 Hz, 9.2 Hz, H-2); 4.32 (dd, 1H, J = 12.4 Hz, 5.3 Hz, H-6a); 4.17 (dd, 1H, J = 12.4 Hz, 2.3 Hz, H-6b); 3.89 (ddd, 1H, J = 12.4 Hz, 5.3 Hz, 2.3 Hz, H-5); 3.30 (s, 3H, CH_3N); 2.13, 2.08, 2.07 (3s, 9H, 3× CH_3).

5.1.19. 3,4,6-Tri-O-acetyl-2-fluoro-2-deoxy-1-(4-methylamino) phenyl-β-D-glucopyranoside, **18**

Compound **18** was prepared according to the procedure described for compound **9a**, starting from compound **17** (258 mg, 0.47 mmol) and 10% Pd/C (52 mg) in THF/MeOH (10/10 mL). The mixture was stirred for 8 h before work-up. The crude product was purified by silica gel chromatography (cyclohexane – EtOAc, 6:4) to give compound **18** (112 mg, 0.27 mmol) as a yellow gum; yield 58%.

^{1}H NMR ($CDCl_3$): δ 6.96 (d, 2H, J = 8.6 Hz, $H_{arom.}$); 6.58 (d, 2H, J = 8.6 Hz, $H_{arom.}$); 5.38 (td, 1H, J = 14.7 Hz, 9.2 Hz, H-3); 5.08 (t, 1H, J = 14.7 Hz, H-4); 4.96 (dd, 1H, J = 7.6 Hz, 2.8 Hz, H-1); 4.52 (td, 1H, J = 50.4 Hz, 9.2 Hz, H-2); 4.29 (dd, 1H, J = 12.5 Hz, 5.2 Hz, H-6a); 4.13 (dd, 1H, J = 12.5 Hz, 2.3 Hz, H-6b); 3.77 (ddd, 1H, J = 12.5 Hz, 5.2 Hz, 2.3 Hz, H-5); 2.82 (s, 3H, CH_3N); 2.10, 2.08, 2.04 (3s, 9H, 3× CH_3).

5.1.20. 1-[N-4-(3,4,6-Tri-O-acetyl-2-fluoro-2-deoxy-β-D-glucopyranosyloxy)phenyl-N-methyl]carbamoyl-5-fluorouracil, **19**

Compound **19** was prepared in two steps according to the procedure described for compound **14a**. First, the carbamoyl chloride was formed starting from **18** (100 mg, 0.24 mmol), $NaHCO_3$ (41 mg, 0.48 mmol), triphosgene (108 mg, 0.37 mmol), and DCM (4 mL). The mixture was stirred for 14 h before work-up to give the carbamoyl chloride of arylamine **18** (91 mg, 0.19 mmol) used without further purification. To a solution of 5-FU (37 mg, 0.29 mmol) in freshly distilled DMAc (2 mL) was slowly added a 60% dispersion of NaH in mineral oil (12 mg, 0.29 mmol) under argon. The mixture was heated at 45 °C for 12 h and the carbamoyl chloride of amine **18** in DMAc (1 mL) was added dropwise. The mixture was stirred for 12 h before work-up to give a residue, which was purified by silica gel chromatography (cyclohexane – EtOAc, 4:6) to give an oil. The latter was triturated in diisopropyl ether (9 mL). Filtration of the precipitate gave compound **19** (39 mg, 0.068 mmol) as a beige powder; mp 84 °C; overall yield 29%.

(−)-ESI-MS: 568.2 $[M - H]^-$.

^{1}H NMR ($CDCl_3$): δ 8.29 (se, 1H, NH); 7.47 (d, 1H, J = 5.0 Hz, 5-FU:H-6); 7.08 (m, 4H, $H_{arom.}$); 5.43 (td, 1H, J = 14.5 Hz, 8.8 Hz, H-3); 5.12 (m, 2H, H-1, H-4); 4.58 (td, 1H, J = 50.6 Hz, 8.8 Hz, H-2); 4.34–4.11 (m, 2H, H-6a, H-6b); 3.90 (m, 1H, H-5); 3.43 (s, 3H, CH_3N); 2.12, 2.08, 2.07 (3s, 9H, 3× CH_3).

^{13}C NMR ($CDCl_3$): δ 170.65, 170.10, 169.62 (3× $OCOCH_3$); 156.69 (5-FU:C-4, $^2J_{C-F}$ = 27.0 Hz); 156.22 ($C_{arom.}$); 156.00 (CH_3NCON); 150.37 (5-FU:C-2); 140.73 (5-FU:C-5, $^1J_{C-F}$ = 240 Hz); 136.78 ($C_{arom.}$); 127.49 ($C_{arom.}$); 125.03 (5-FU:C-6, $^2J_{C-F}$ = 34.3 Hz); 127.49 ($C_{arom.}$); 98.40 (C-1, $^2J_{C-F}$ = 23.6 Hz); 89.10 (C-2, $^1J_{C-F}$ = 191 Hz); 72.60 (C-3, $^2J_{C-F}$ = 19.8 Hz); 72.22 (C-5); 67.99 (C-4, $^3J_{C-F}$ = 7.1 Hz); 61.79 (C-6); 39.83 (NCH_3); 20.86, 20.75, 20.65 (3× $OCOCH_3$).

5.2. HPLC-UV analysis

A new HPLC-UV method was developed to simultaneously analyze 5-FU and its derivatives. An HP 1050 (Agilent, palo Alto, CA, USA) liquid chromatograph equipped with a 150 × 3.0 mm

Phenomenex Luna PFP(2) reversed-phase column (Le Pecq, France), particle size 3 μm, pore size of 100 Å was used for the overall analysis.

The solvents used to prepare the mobile phase were 10 mM ammonium formate buffer, adjusted to pH 3 with formic acid and filtered through a 0.45 mm filter (Millipore, Saint-Quentin, Yvelines, France) (component A) and acetonitrile (component B). The initial mobile phase composition was 0% B, which was held for 6 min after injection of sample. A linear gradient was then started in which the proportion of B increased from 0% to 85% in 14 min. Eventually the column was re-equilibrated at 0% B from 25 min to 35 min. The total run time was 35 min. HPLC separations were performed at room temperature at a flow rate of 0.4 mL/min and an injection volume of 10 μL. Detection of compounds was performed using a UV detector: at 264 nm from 0 min to 7 min and then at 258 nm.

5.3. Characterization of degradation products by HPLC-UV-MS

The type of column and the mobile phase composition were the same as described above. The mass spectral data were acquired by electrospray ionization mass spectrometry (ESI-MS) on a TSQ7000 mass spectrometer from Thermo Finnigan (San José, CA, USA). The detection was carried out by positive or negative electrospray ionization and the source parameters were tuned as follows: spray voltage, 4.5 kV; capillary temperature, 350 °C; nitrogen sheath gas, 80 Psi; nitrogen auxiliary gas, 40 AU; argon collision pressure, 2.5 mTorr. The data acquisition was performed with the Xcalibur® 1.1 software package from Thermo Finnigan. The mass range scanned in MS runs was m/z 50–800. Each MS spectrum was recorded by averaging 20 spectra.

MS analyses were performed using total ion current (TIC) chromatograms. Specific compounds (m/z corresponding to prodrugs and/or specified degradation products) were detected using extracted ion current (EIC) chromatograms.

5.4. Sample preparation

The stability studies of potential prodrug in solution were conducted at 37 ± 2 °C. A stock solution of each compound was prepared in methanol or in a mixture of methanol and acetonitrile (85:15, v:v) to give a final concentration of 10 mM. From this stock solution, a 1:10 dilution was prepared in the relevant media (PBS (Gibco), MEM or human plasma) in 7 mL glass tubes. The solution was maintained at 37 ± 2 °C in an oven, and aliquots (500 μL) were withdrawn at various time points, quenched with an equal volume of ammonium formate buffer 10 mM pH 3 and vortexed for 1 min. After centrifugation at 3200 r.p.m. for 10 min, an aliquot of supernatant was filtered through a 0.45 μm PVDF filter (AIT, France) and diluted (3:10) in 10 mM ammonium formate buffer. The mixture was vortexed and analyzed by the chromatographic method described above. The disappearance of the potential prodrug was followed by HPLC-UV analysis. All the experiments were carried out in triplicate unless otherwise stated.

5.5. Data analysis

The time-dependent decline in the concentrations of the potential 5-FU derivatives after incubation in various media was fitted using a first-order kinetics model, and their degradation half-lives were estimated from the slopes of fitted lines.

5.6. Biological assays

5.6.1. Cell culture

Normal human fibroblasts were purchased from Promocell (Heidelberg, Germany). This frozen culture was obtained from foreskin waste from a 6-year old Caucasian male and the cells used in this work were from the seventh to twelfth passage of the culture. Breast cancer adenocarcinoma MCF 7 and ovary teratocarcinoma PA 1 human cell lines were purchased from the European Collection of Cell Cultures (ECACC; Salisbury, United Kingdom). Stock cell cultures were maintained as monolayer in 75 cm^2 culture flasks in Glutamax™ Eagle's Minimum Essential Medium with Earle's salts (MEM; Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal calf serum (Sigma, St Quentin Fallavier, France), 1 mM sodium pyruvate (Invitrogen), 1X vitamins solution (Invitrogen), 1X non essential amino acids solution (Invitrogen) and 4 μg/mL of gentamicine (Invitrogen). Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

5.6.2. Cell growth inhibition assay

Cells were plated at a density of 5×10^3 cells per well in 96-well microplates (Nunclon™, Nunc, Roskilde, Denmark) in 150 μL of culture medium and were allowed to adhere for 16 h before treatment with the compound tested. Stock solution of each compound was prepared in dimethylsulfoxide (DMSO) and kept at −20 °C until use. The percentage of DMSO was kept at 0.5% (v/v) whatever the concentration tested. This percentage did not modify cellular growth. Fifty microliters of a 4X solution in MEM were then added and a 48 h continuous drug exposure protocol was used. Then, the cytotoxic effect of compounds on tumor cells was assessed by using Resazurin reduction test.

5.6.3. Resazurin reduction test

The resazurin reduction test (RRT) was carried out according to the protocol described previously [38]. Briefly, plates were rinsed by 200 μL PBS (37 °C, Invitrogen) using a multichannel dispenser (Labsystems, Helsinki, Finland) and emptied by overturning on absorbent toweling. Then, 150 μL of a 25 μg/mL solution of resazurin in MEM without SVF or Phenol red was added in each well. The plates were incubated 1 h at 37 °C in a humidified atmosphere with 5% of CO_2 for fluorescence development by living cells. Fluorescence was then measured on the automated 96-well plate reader Fluoroskan Ascent FL™ (Labsystems) using an excitation wavelength of 530 nm and an emission one of 590 nm. The fluorescence is proportional to the number of living cells in the well and IC_{50} (drug concentration required to decrease final cell population by 50%) was calculated from the curve of concentration-dependent cell number decrease, defined as the fluorescence in experimental wells as a percentage of that in control wells, with blank values subtracted.

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