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Synthesis and cytotoxic properties of new fluorodeoxyglucose-coupled chlorambucil derivatives

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Abstract—Frequently used in the treatment of malignant cells, alkylating agents, like most anticancer substances, produce adverse side effects caused by the toxicity of the agents toward normal tissues and lose efficiency through poor distribution to target sites. Our approach to developing more selective drugs with low systemic toxicity is based on the premise that the body distribution and cell uptake of a drug can be altered by attaching a neoplastic cell-specific uptake enhancer, such as 2-fluoro-2-deoxyglucose (FDG), the radiotracer most frequently used in PET for tumor imaging. Two properties of deoxyglucose, namely preferential accumulation in neoplastic cells and inhibition of glycolysis, underpin this targeting approach. Here, we report the synthesis of 19 new chlorambucil glycoconjugates in which the alkylating drug is attached to the C-1 position of FDG, directly or via different linkages. This set of compounds was evaluated for in vitro cytotoxicity against different human normal and tumor cell lines. There was a significant improvement in the in vitro cytotoxicity of peracetylated glucoconjugates compared with the free substance. Four compounds were finally selected for further in vivo studies owing to their lack of oxidative stress-inducing properties.

1. Introduction

Selectively directing a drug into the vicinity of its intended site of action has clear therapeutic advantages, including reduced toxicity, smaller dose levels and especially enhanced efficacy. This is particularly true in cancer chemotherapy, where most of the pharmaceuticals currently in use do not accumulate selectively in malignant tissues or cells. The main limitations in conventional cancer chemotherapy arise from the biodistribution of cytotoxins in the body; lack of drug-specific affinity toward tumor cells, need for a large total dose of a drug to achieve high local concentration and systemic toxicity leading to many negative, sometimes life-threatening, side effects. Acute toxicity affects rapidly dividing normal cells such as bone marrow cells and intestinal epithelial cells. Non-selective cytotoxicity is the main effect that limits the use of optimal doses in most conventional chemotherapeutic drug regimens. Consequently, protocols with sub-optimal doses are frequently used, minimizing toxicity and patient suffering but often resulting in inefficient treatment and unsatisfactory prognosis (residual disease, tumor regrowth, metastasis, drug resistance, etc.). This major issue dictates research in selective chemotherapy by drug targeting—if a drug can be selectively targeted to a tumor to provide a high concentration of drug selectively in the target zone, with less drug reaching critical normal tissues, then many problems associated with the systemic toxicity of chemotherapy will be resolved. Cancer therapy and patients' quality of life can thereby be substantially improved.

Several reviews have examined new drug delivery systems (DDSs) to achieve drug targeting.[1–3] DDS are based on two main principles: (i) passive targeting, using a vehicle, and (ii) active targeting, using a specific vector and exploiting an increased affinity of the delivery system toward malignant cell or tissue components. The first approach involves the development of

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reservoir-type systems to encapsulate a drug,[4,5] such as polymers, microcapsules, nanoparticles, and micellar or liposomal drug preparations, which cause spontaneous drug accumulation due to enhanced permeability and retention effects.[6] The second approach uses a tumor characteristic: a drug is conjugated with a suitable vector to impart specific affinity for the target to a non-specific drug.[7] Active DDSs offer a wide scope using various kinds of carriers, such as antibodies, peptides, proteins (lectins,[8] albumins), hormones, charged entities, mono-, oligo- and polysaccharides,[9] and some low molecular-weight ligands, such as folate or lactose,[10] for example. The two approaches can be combined using DDS nanoparticles bearing active carriers.[11]

Active targeting is largely concerned with immunoconjugates[12] especially in antibody-directed enzyme prodrug therapy (ADEPT).[13,14] The application of monoclonal antibodies for tumor targetability of chemotherapeutics appears promising. However, it will be restricted to tumors expressing high levels of related antigen. Our work is part of another strategy using low molecular-weight ligands.

Previous work by our team has shown that drugs can be targeted toward particular types of tumors, using biochemical characteristics specific to these cells, such as high melanin content in melanoma[15] or the presence of proteoglycans in chondrosarcoma.[16,17] However, more ubiquitous drug delivery systems aiming at solid tumors could extend this type of approach to a much greater number of cancers, given that solid tumors represent 89% of malignancies. To achieve this ubiquitous targeting, we have chosen to exploit a well-documented, typical biochemical phenotype of all invasive solid tumors that confers a selective growth advantage: the upregulation of glycolysis, resulting in increased rates of glucose consumption compared with normal, non-transformed cells.[18] It has long been recognized that malignant cells most often exhibit increased glucose metabolism[19] and increased glucose transport.[20] This upregulation of glycolysis observed especially in neoplastic tissues is nearly ubiquitous in cancers and increases with tumor aggressiveness. Even under aerobic conditions, neoplastic cells need a 19-fold increase in glucose consumption per mole of ATP produced because they lose efficient production of ATP by the aerobic oxidative phosphorylation in mitochondria, and therefore rely solely on glycolysis to provide the energy requirements of rapidly replicating tissue. This leads to progressive increases in glucose uptake with malignant evolution.

Thus, glycolysis-mediated targeting using a glucose derivative as an active drug carrier could be useful for delivering chemotherapy selectively to cancer cells. Studies aimed at increasing tumoral selectivity by conjugation of therapeutics to sugars have already been undertaken.[21–23] One of the first anticancer agents designed to exploit the upregulation of glycolysis and successfully tested in clinical trials[24] is the new drug glufosfamide, a sugar-linked isophosphoramide mustard derivative exploiting transmembrane glucose transport.[25,26] Glufosfamide shows lower medullary toxicity and greater antitumor activity than ifosfamide. These improvements are due to increased uptake of the agent by the tumoral cells due to the glucose moiety.[27,28] Work on the affinity of some new antineoplastic compounds for the GLUT1 carriers have also been published; these new compounds, such as hexose keto-C-glycoside conjugates,[29] glucose–chlorambucil derivatives[30] and *O*-methylsulfonyl derivatives of glucose[23] were designed to enhance selectivity for brain tumors. However, none of these studies has produced a therapeutic drug.

To use the strongly increased uptake of sugars by tumors, and also to potentiate the cytotoxic effect of the chemotherapeutic agent carried, we chose a particular glucose derivative to act as selective drug carrier, 2-deoxy-2-fluoro-D-glucose (FDG). FDG is a structural analogue of glucose, differing at the second carbon atom by the substitution of a fluoro for a hydroxyl group. Its radiolabeled fluorine-18 derivative[31] is the main tumor-specific radiopharmaceutical agent used in tumor imaging by positron emission tomography (PET).[32] Widespread clinical use of ^{18}F-FDG PET has unequivocally demonstrated that most primary and metastatic human tumors avidly trap FDG to meet the increased glucose. Hence the use of an FDG moiety coupled to a chemotherapeutic agent should in principle make it possible to trap the glycoconjugate inside the tumor cells.

In addition, nutrient deprivation has been shown to cause cancer cell death, and some glucose analogues, such as deoxyglucose (DG), can mimic glucose deprivation by profoundly inhibiting glucose metabolism. Some data suggest that neoplastic cells treated with DG accelerate their own demise[33–35] and that DG[36–38] and its tetraacetate ester[39] enhance the cytotoxic effects of anticancer agents. Normal and transformed cells respond to glucose antagonists in opposing manners. FDG bearing a chemotherapeutic may thus interfere with glucose metabolism like DG, and sensitize tumoral cells to the cytostatic action of the FDG conjugates.

In this respect, there are two main mechanisms whereby FDG-cytotoxic conjugates may prove advantageous: (i) increased glycolysis to support the metabolic requirements of malignant cells results in high glucose consumption and may lead to enhanced intracellular uptake of fluoroglycosylated drugs in tumor cells compared with healthy ones and (ii) DG interferes with energy metabolism, resulting in cancer cell death by apoptosis.

These properties of FDG recently prompted us to initiate a new tumor-targeting approach, using FDG as a drug carrier.

Chlorambucil (CLB) is a DNA-alkylation-inducing nitrogen mustard currently used in clinical treatment especially against chronic lymphatic leukemia, lympho-

mas, and advanced ovarian and breast carcinomas. Chemotherapy protocols using CLB are limited by toxicity with nausea, myelotoxicity and neurotoxicity. Like all alkylating agents, its toxicity arises from its non-specificity for tumor tissue when administered systemically and from its oxidative stress-inducing properties in normal cells. In the present study, chlorambucil was used as a model drug to develop FDG-glycolysis-mediated targeting. Here, we describe the synthesis, characterization and in vitro cytotoxicity of new glycoconjugates in which the dialkylating antitumor compound chlorambucil is attached to the C-1 position of the FDG skeleton using a variety of linkages.

The introduction of a spacer arm between the carrier and the drug is a strategy widely used to separate the active moiety from the carrier. Ideally, as a conjugate is meant to reach the malignant site, the linkage should be sufficiently stable during circulation in blood stream to maintain its chemical integrity until it reaches its target. However, after uptake into the cells, they should have an intrinsic activity or release the active compound.

There are many ways to tether a drug to the carrier; however, the carrier must always be anchored in a manner that assures successful targetability. Considering that many times, if the drug molecule is positioned too closed to the carrier, the overall affinity of the conjugate for the targeted receptor or the accessibility of the cytotoxic drug to its final destination could be reduced or even ablated. Admittedly, steric interference is more pronounced for small ligands (pteroates, amino acids, sugar, etc.) than it is for larger ligands (protein, MAb, polymer, nanoparticules, etc.). Thereby, initially in order to evaluate the necessity of spacing between CLB and the sugar carrier, we synthesized compounds **14**, **15**, and **17** where CLB was directly linked to the FDG moiety through an ester, amide, or urea function. Then, following the widely applied strategy, we also prepared compounds with a spacer arm between the fluorosugar moiety and CLB; the composition of the linker can vary widely and the linker can be designed to contain stable or cleavable functional groups, and at the same time residues that may influence the physical properties such as the hydrophilic-lipophilic balance. Indeed, depending on the nature of the drug activity, it may be crucial to have it dissociated from the carrier following endocytosis. In our case, owing to CLB cancer cell-killing mechanism through DNA alkylation, the overall carrier-drug conjugate or chlorambucil derivatives with 'chemical baggage' could be even cytotoxic. Thus, we decided to use linkers possessing cleavable bond juxtaposed to the drug to allow the release of the original active chemical form (CLB) at the opposite extremity of the linker and/or at the level of the spacer-backbone bond, so that spacer-CLB moieties can be similarly released and also have intrinsic activity as nitrogen mustard.

Many types of functions can be employed to assure the in vivo cleavage which can be due to passive hydrolysis or caused by a more specific mode: enzymatic, reductive or pH-controlled release.[40,41] CLB providing a carboxyl group is appropriately functionalized to bind to the spacer arm or to the FDG residue by the formation of an ester or an amide bond, also we choose in the present work preferentially ester and amide functions, two of the most-used bonds between a drug and the labile residue in targeted drug strategies.[30,42–47] Enzymatic and non-enzymatic hydrolysis of ester and amide functionality has been most often reported. For many drugs, passive hydrolysis may occur in aqueous media; the rate of release will decrease in the order ester > amide.[40]

The structure of all the targeted CLB–FDG conjugates varying by the nature of the spacer is given in Figure 1.

2. Results and discussion

2.1. Chemistry

Schemes 1–6, describe the syntheses of compounds with linker modifications. 1,3,4,6-Tetra-*O*-acetyl-2-deoxy-2-fluoro-β-D-glucopyranose **5** and its derivatives **6**–**13** (Scheme 1) are the key intermediates for all the syntheses in which chlorambucil bonds directly or via a spacer to C-1 of the sugar moiety. Compound **5** was prepared according to the method described by Kovac.[48] The first step of the reaction involved the conversion of D-mannose **3** into 1,3,4,6-tetra-*O*-acetyl-β-D-mannopyranose **4** and is a modification of the procedure described by Deferrari et al.[49] The second step gave compound **5** by introducing a fluorine atom at C-2 of the D-mannose derivative, using diethylaminosulfur trifluoride.[48] The introduction of a spacer via a glycolytic or amide function under Koenigs–Knorr conditions needs the synthesis of compound **6**, which was achieved with hydrobromic acid by the procedure described by Baer et al.[50] The NMR data for this compound agree with those already published.[51]

The coupling of a moiety at C-1 of a sugar via an ester bond required the preparation of compound **9**, while an amide bond needed the synthesis of the amino derivative **8**, which was obtained from the α-bromo compound **6** through the β-anomeric azide **7**. The α,β-glucopyranose **9** was obtained in quantitative yield from chemoselective deacetylation of the peracetylated **5** with ammonium carbonate in DMF.[52]

For the preparation of compounds possessing an ester linkage between the active drug and the anchor point of the carrier, the use of hydroxyl protecting groups other than acetates proved necessary. Benzyl ethers were chosen. These benzyl-protected sugars were obtained starting from bromo-sugar **6**. This compound was treated with sodium ethanethiolate to give the ethyl β-thioglycoside **10**[53] in 87 % yield. Replacement of the acetate protecting group with benzyl ethers was achieved in two steps: removal of acetyl groups by Zemplèn[54] transesterification followed by treatment with benzyl bromide in DMF in the presence of sodium hydride. The addition of bromide to thioglyoside **11** gave the newly benzyl-protected bromoglycoside **12**.[53] The 3,4,6-tri-benzyl-2-deoxy-2-fluoroglucose **13** was synthesized from 3,4,6-tri-benzyl-2-deoxy-2-fluoroglucopyranosyl bromide **12** using silver carbonate and water.

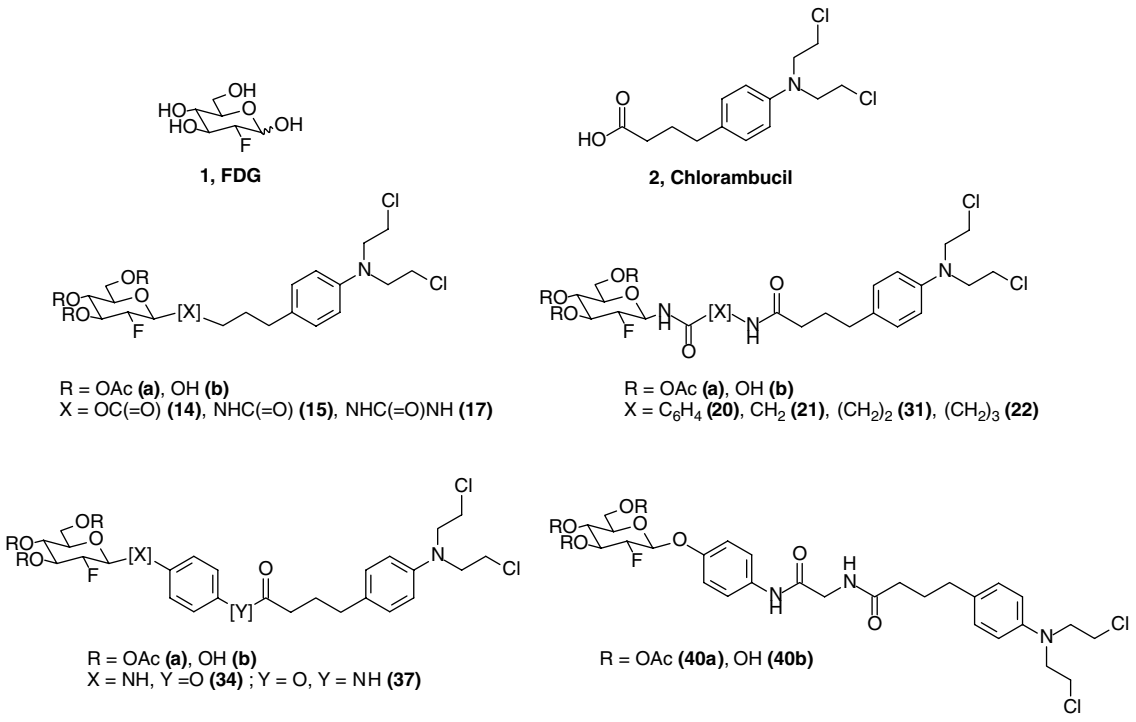


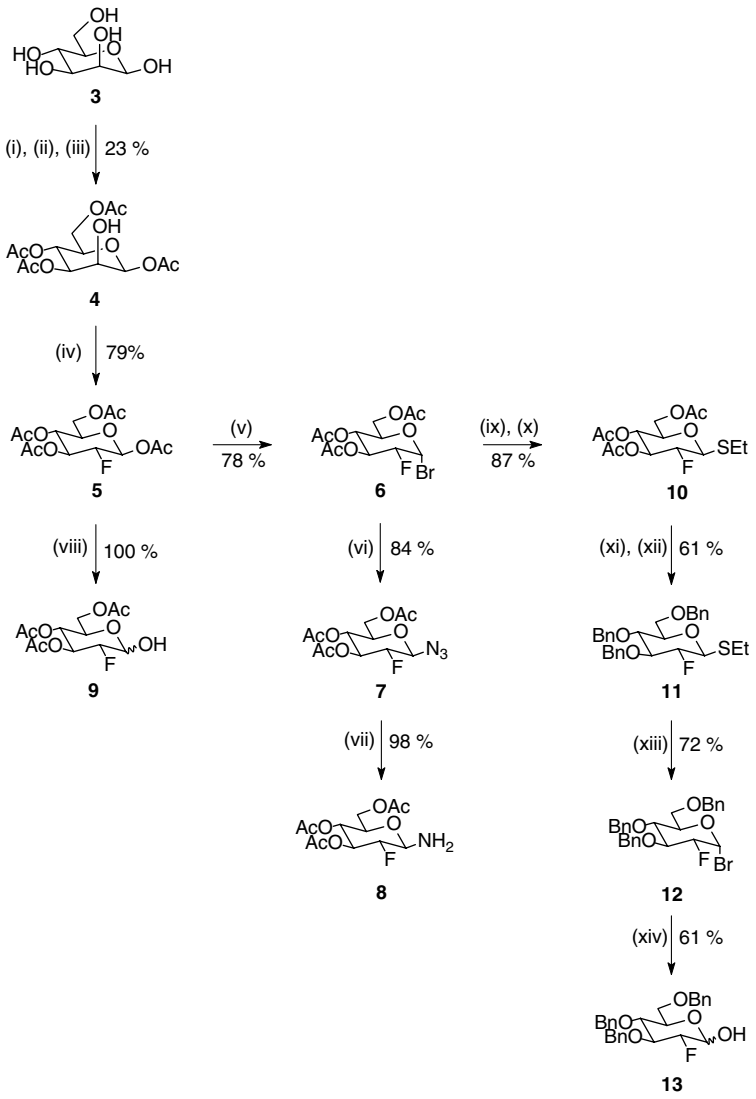
Figure 1. Structures of FDG, chlorambucil and all target compounds.

To evaluate the importance of the type of linkage binding the carrier and the drug, we synthesized compounds **14**, **15**, and **17**, where the active molecule was directly linked to the FDG moiety through an ester, amide, or urea function (Scheme 2).

The reaction of **8**, **9**, and **13** with chlorambucil, activated by the *N*,*N*′-dicyclohexylcarbodiimide (DCC) procedure, using either 4-(*N*,*N*-dimethylamino)pyridine (DMAP) or hydroxybenzotriazole (HOBt), gave the corresponding esters **14** and amide **15**. Using an ester linkage between the sugar and chlorambucil, the hydroxyl compound **14c** cannot be obtained from the peracetylated one **14a**, the linkage being cleaved during the Zemplèn deacetylation. Accordingly, **14c** was prepared from the benzyl derivative **14b** after hydrogenolysis (H_2, Pd/C, quantitative). The acetylated compound **17a** was obtained from chlorambucil in two steps including the formation of the isocyanate **16** and its reaction with β-amino-sugar **8**. To synthesize isocyanate **16**, sodium azide, ethylchloroformate and triethylamine were first added to chlorambucil and gave the corresponding acyl azide. Subsequent heating produced, after Curtius rearrangement, isocyanate **16** in 81% yield. The removal of the acetyl groups of glycoside **17a** gave the expected compound **17b** in only 45% yield. Derivatives **15** and **17** were enantiomerically pure and only obtained as the β form. Compounds **14a**, **14b**, and **14c** were isolated as an inseparable mixture of the two anomers with α/β ratios of, respectively, 25:75, 85:15, and 55:45. The ratio was calculated from their ^{1}H NMR spectra: for example, H-1 of the α-anomer **14c** resonates as a doublet at 6.41 ppm ($J_{1,2}$ = 3.9 Hz), whereas H-1 of the β-anomer shows an extra coupling with the C-2 fluorine atom at 5.85 ppm and resonates as a doublet of doublets ($J_{1,2}$ = 8.1 Hz, $J_{1,F}$ = 3.1 Hz).

For the preparation of compounds **20b**, **21b** and **22b** for which 4-aminobenzamide, 2-aminoethanamide or 4-aminobutanamide were used, respectively, as spacer arms between the sugar residue and chlorambucil, the synthetic methods in Scheme 3 were adopted. The N-acylation of the β-aminosugar **8** with 4-nitrobenzoyl chloride, *N*-Cbz-glycine or 4-azidobutanoic acid **25** led, respectively, to derivatives **18a**, **18b**, and **18c** in good yields. The requisite azido acid **25** was readily accessible in almost quantitative yield from the commercially available 4-bromobutanoic acid ethyl ester **23** through a two-step sequence: the formation of the corresponding azido ester **24** by the substitution of bromide using sodium azide and hydrolysis of the ethyl ester with aqueous potassium hydroxide. Either the reduction of the nitro or the hydrogenation of azido function or the cleavage of the benzyloxycarbonyl (Cbz) protecting group in compounds **18a–c** by hydrogen provided the corresponding amino derivatives **19a–c**. These intermediates were then directly *N*-acylated with chlorambucil activated by DCC and HOBt or by ethylchloroformate and triethylamine, leading to enantiomerically pure compounds **20a, 21a**, and **22a**. Final deacetylation by the Zemplèn method using sodium methoxide in methanol afforded the fully deprotected species **20b**, **21b**, and **22b** (enantiomerically pure as shown by NMR spectra) in good yields. NMR and MS data agree with the proposed structures.

Compounds **31** were to be obtained in a similar way as for derivatives **20**, **21**, and **22**, but the synthetic sequence required the preparation of 3-azidopropionic acid. De-



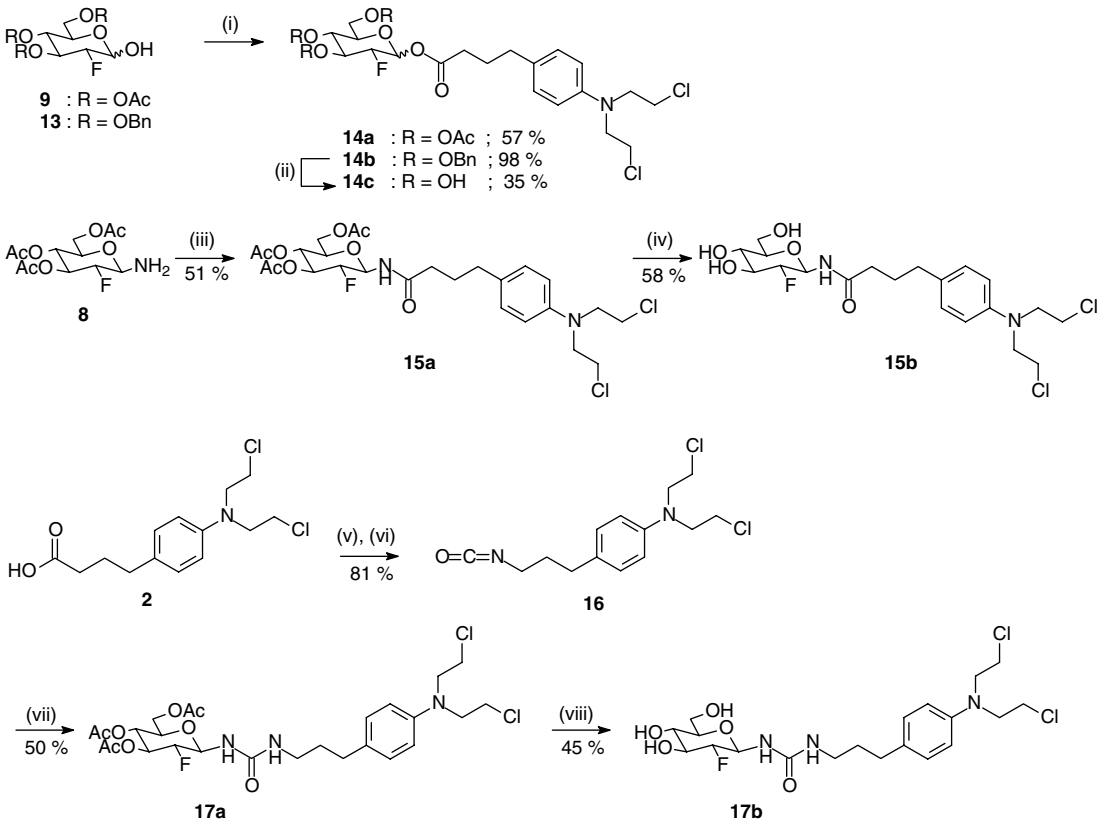
Scheme 1. Reagents and conditions: (i) Ac_2O, $HClO_4$; (ii) PBr_3, H_2O; (iii) AcONa, H_2O; (iv) DAST, dioxane; (v) HBr/AcOH, $CHCl_3$; (vi) NaN_3, acetone/H_2O; (vii) H_2, Pd/C, THF/MeOH; (viii) $(NH_4)_2CO_3$, DMF; (ix) EtSH, NaOMe; (x) Ac_2O, AcONa; (xi) NaOMe, MeOH; (xii) BnBr, NaH, KI; (xiii) Br_2, Et_2O; (xiv) Ag_2CO_3, H_2O/acetone.

spite repeated attempts, this compound could not be obtained pure and so another reaction pathway (Scheme 4) was used, with preliminary coupling of chlorambucil with the spacer before the N-glycosylation, in contrast to the other pathway.

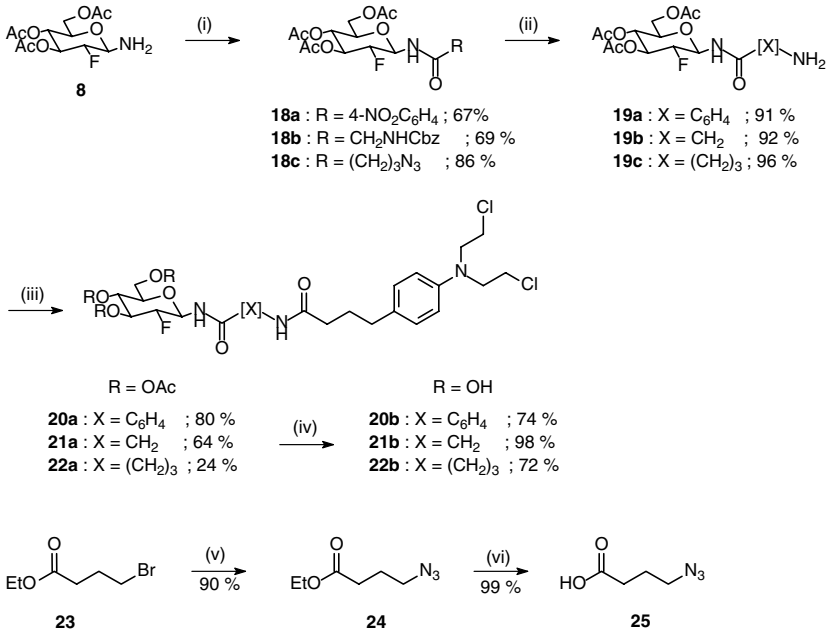
Starting from 3-bromopropionic acid ethyl ester **26**, the substitution of bromide using sodium azide followed by hydrogenolysis in the presence of Pd/C provided 3-aminopropionic acid ethyl ester **28**. N-Acylation of the latter with chlorambucil (C1COOEt, Et_3N, CH_2C1_2) gave the amide **29**. After hydrolysis to the carboxylic acid **30**, the *N*-glycoside **31a** was prepared from the amino-sugar **8** under N-acylation conditions. Final deacetylation gave the target compound **31b** in 90% yield.

We also prepared compounds **34** and **37** in which 4-aminophenol was used as a spacer between the sugar moiety and chlorambucil (Schemes 5 and 6). The introduction of this moiety can lead to an *N*-glycoside **34** with chlorambucil anchored to the spacer through an ester bond, or to an *O*-glycoside **37** with chlorambucil anchored through an amide bond. Reaction of chlorambucil with 4-nitrophenol, using DCC and DMAP as condensing reagents, gave the ester **32** in good yield. Hydrogenation of **32** catalyzed by Pd/C afforded the reductive analogue **33**. The protected β-glucopyranose group was introduced under Koenigs–Knorr conditions by N-glycosylation of the aniline **33** with 3,4,6-tri-benzyl-2-deoxy-2-fluoroglucopyranose bromide **12** in the presence of silver oxide as catalyst. The stereochemistry of **34a** was assigned based on the multiplicity of H-2 observed in ^{1}H NMR: H-2 resonates at 4.41 ppm as a doublet of triplets ($J_{2,1} \approx J_{2,3} = J_{ax-ax} = 7.5$–$8.5$ Hz, $J_{2,F} = 51.0$ Hz). Hydrogenolysis of **34a** (H_2, Pd/C, EtOH) then gave **34b**.

The glycosylation to **35** was conducted under Koenigs–Knorr conditions by the reaction of bromide **6** with 4-nitrophenol in the presence of silver oxide in acetoni-

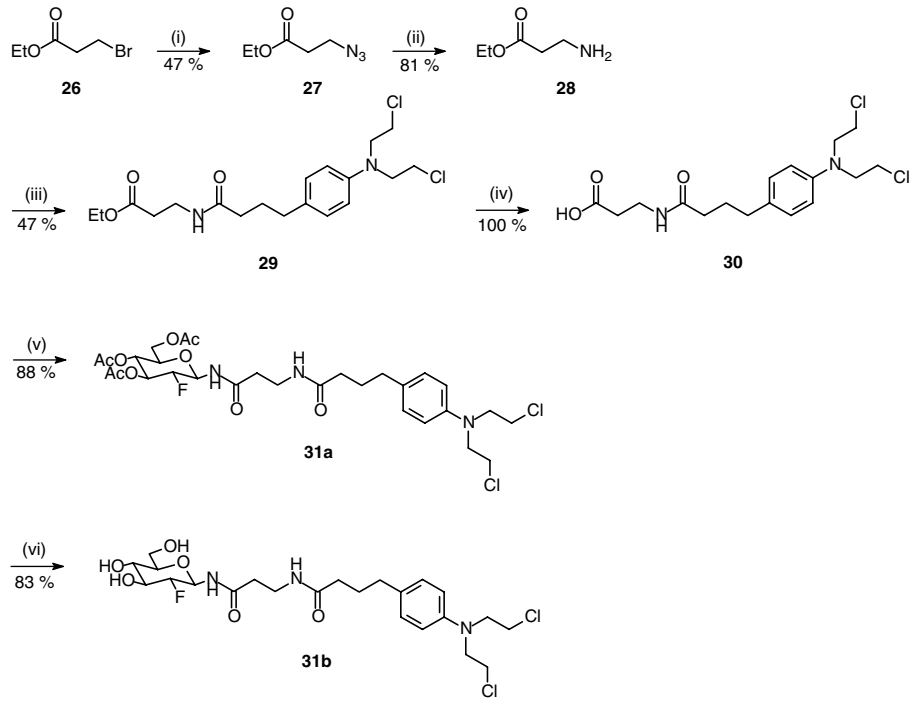


Scheme 2. Reagents and conditions: (i) chlorambucil, DCC, DMAP, CH_2Cl_2; (ii) $HCOONH_4$, Pd/C, THF/EtOH; (iii) chlorambucil, DCC, HOBt, DMF; (iv) NaOMe, MeOH; (v) ClCOOEt, Et_3N, NaN_3, acetone; (vi) toluene, reflux; (vii) **8**, CH_2Cl_2/THF; (viii) NaOMe, MeOH.

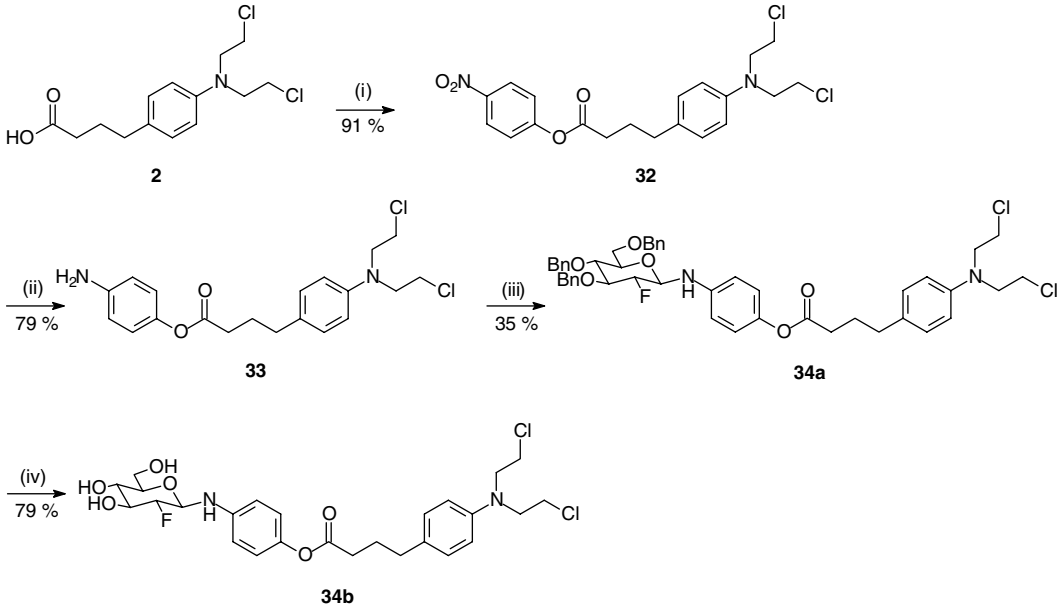


Scheme 3. Reagents and conditions: (i) **18a**: 4-nitrobenzoyl chloride, Et_3N, CH_2Cl_2; **18b**: *N*-Cbz-glycine, DCC, HOBt, DMF; **18c**, **25**: ClCOOEt, Et_3N, CH_2Cl_2; (ii) H_2, Pd/C; (iii) **20a**, **22a**: chlorambucil, ClCOOEt, Et_3N, CH_2Cl_2; **21a**: chlorambucil, DCC, HOBt, DMF; (iv) NaOMe, MeOH; (v) NaN_3, acetone/H_2O, reflux; (vi) KOH, MeOH/H_2O.

trile. After stirring for one day at room temperature, the silver salts were filtered off and chromatography on silica gel afforded β-anomer **35** exclusively, in good yield. The stereochemistry of **35**, similar to that of **34a**, was assigned based on its ^1H NMR signature and particularly on the multiplicity at and near the anomeric center: H-1



Scheme 4. Reagents and conditions: (i) NaN_3, $MeOH/H_2O$; (ii) H_2, Pd/C, MeOH/THF; (iii) chlorambucil, ClCOOEt, Et_3N, CH_2Cl_2; (iv) LiOH, $EtOH/H_2O$; (v) **8**, ClCOOEt, Et_3N, CH_2Cl_2; (vi) NaOMe, MeOH.

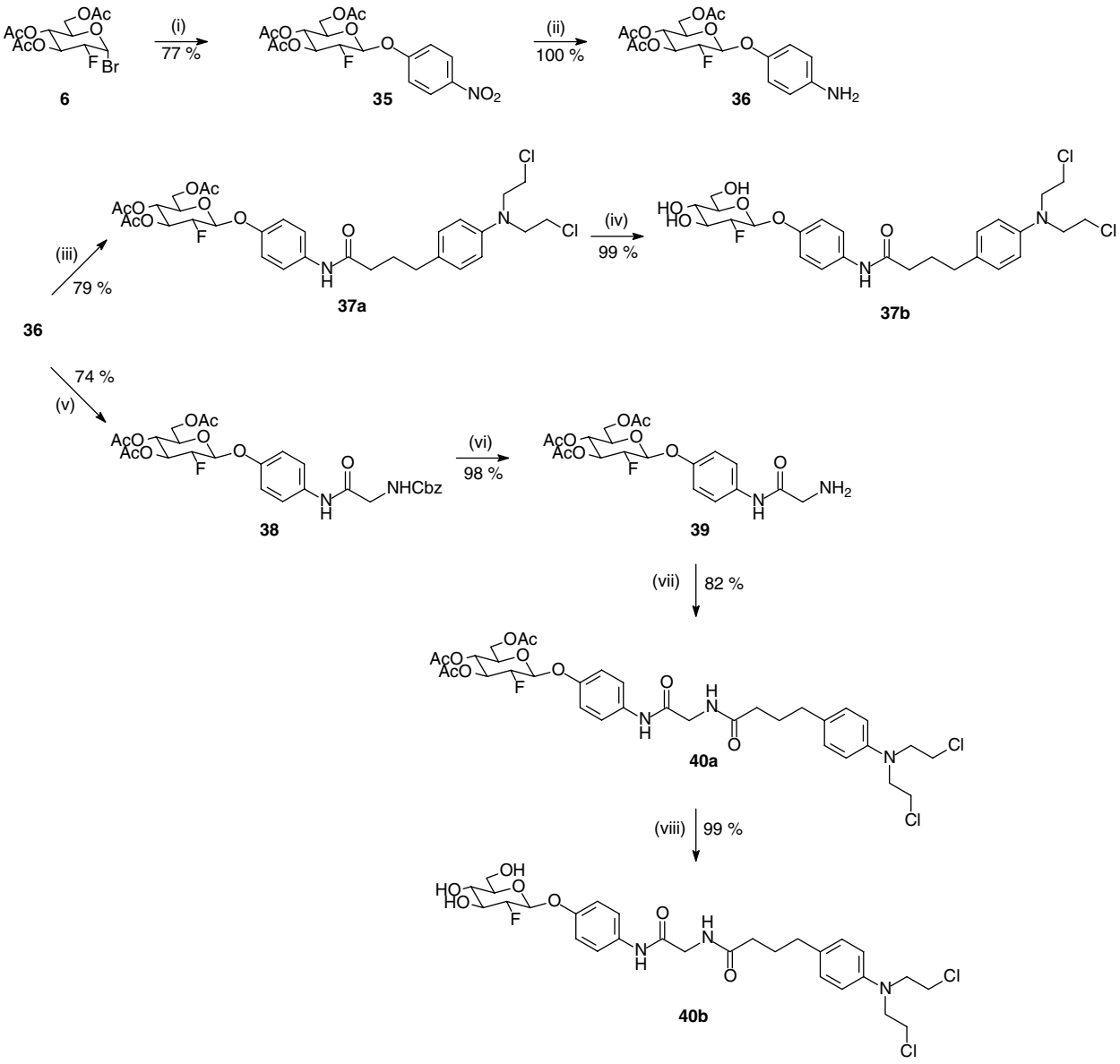


Scheme 5. Reagents and conditions: (i) 4-nitrophenol, DCC, DMAP, CH_2Cl_2; (ii) H_2, Pd/C, EtOH; (iii) **12**, Ag_2O, CH_3CN; (iv) H_2, Pd/C, EtOH/THF.

resonates at 5.30 ppm as a doublet of doublets ($J_{1,2} = J_{ax-ax} = 7.6$ Hz, $J_{1,F} = 3.1$ Hz) and H-2 resonates at 4.63 ppm as a doublet of doublets of doublets ($J_{2,3} = J_{ax-ax} = 9.2$ Hz, $J_{2,F} = 50.4$ Hz). Reduction of the nitro group was achieved by hydrogenation to give derivative **36**. Thus, the compound was used as a reagent for the two different syntheses leading to derivatives **37** and **40**.

N-Acylation of amine **36** with activated chlorambucil (DCC-HOBt/DMF) gave compound **37a**. Deacetylation then gave the expected compound **37b** in quantitative yield.

Finally, we describe the synthesis of glycoside **40** for which a glycine moiety was added next to the aminophenol to provide the spacer domain. Starting from aniline



Scheme 6. Reagents and conditions: (i) 4-nitrophenol, Ag_2O, CH_3CN; (ii) H_2, Pd/C, EtOH/THF; (iii) chlorambucil, DCC, HOBt, DMF; (iv) NaOMe, MeOH; (v) *N*-Cbz-glycine, DCC, HOBt, DMF; (vi) $HCOONH_4$, Pd/C, EtOH/THF; (vii) chlorambucil, DCC, HOBt, DMF; (viii) NaOMe, MeOH.

36, DCC/HOBt coupling to *N*-Cbz-glycine preceded hydrogenolytic removal of the benzyl carbamate to give **39**. N-Acylation with chlorambucil afforded the acetyl derivative **40a** and Zemplèn deprotection gave the expected compound **40b**. NMR and mass spectrometry confirmed the structure and purity of the pure enantiomers **37** and **40**.

2.2. Biological activity

2.2.1. In vitro cytotoxicity. The cytotoxic activities of the compounds were evaluated in vitro against the six human tumor cell lines; breast adenocarcinoma MCF-7, colon adenocarcinoma DLD-1, ovary adenocarcinoma PA1, prostatic adenocarcinoma PC3, lung carcinoma A549, melanoma M4Beu and a primary culture of human fibroblasts. The cytotoxicity results for some target compounds and chlorambucil are summarized in Table 1. IC_{50} values revealed that the peracetylated compounds (**15a**, **17a**, **20a**, **21a**, **22a**, **31a**, **37a**, and **40a**) were more potent than chlorambucil whatever the cell line considered. Most often the peracetylated compounds showed a low IC_{50} value of 15 μM, whereas CLB per se is only moderately cytotoxic in these conditions (IC_{50} values above 40 μM except in PA1 cells, a very sensitive cell line). Corresponding unprotected derivatives showed a drop in activity (results not shown). This observation had already been made on a series of hexose keto-C-glycoside conjugates[55] which prevent the uptake of [^{14}C]-glucose by human GLUT-1 glucose transporter, and the authors connect this effect to lipophilicity. Considering compounds **15a** and **17a** varying only by one connection, an amide or an urea function linking directly the FDG moiety and CLB, no difference on cyto-

Table 1. Cytotoxicity induced by the new synthesized compounds on human normal and tumor cells

Compound	IC_{50} (μM)						
	Fibroblasts	PC3	MCF7	A549	DLD1	M4Beu	PA1
15a	11 ± 4	4.4 ± 0.9	27 ± 7	5.4 ± 0.4	7.1 ± 0.9	27 ± 9	1.3 ± 0.2
17a	13 ± 2	4 ± 1	26.4 ± 0.6	8 ± 2	7 ± 3	27 ± 11	0.6 ± 0.2
20a	4 ± 2	1.4 ± 0.3	5 ± 2	2.7 ± 0.6	3 ± 2	7 ± 3	0.3 ± 0.1
21a	4.1 ± 0.1	10 ± 2	8 ± 2	5 ± 2	4 ± 3	6.8 ± 0.9	1.0 ± 0.1
22a	4.1 ± 0.1	3 ± 1	12 ± 1	13 ± 5	9 ± 2	9 ± 3	0.27 ± 0.04
31a	7 ± 5	3.7 ± 0.3	13 ± 5	9.2 ± 0.5	11 ± 4	15 ± 3	0.24 ± 0.08
37a	>50	11 ± 4	26 ± 11	1.90 ± 0.01	10 ± 5	>50	0.76 ± 0.06
40a	16 ± 5	8 ± 3	14 ± 5	10 ± 4	6 ± 2	14 ± 5	1.6 ± 0.5
CLB	99 ± 2	nd	>100	43 ± 14	47 ± 17	>100	2.8 ± 0.4

toxicity was observed. Substances possessing two amidic functions correspond to the most cytotoxic compounds namely **20a**, **21a**, **22a**, **31a**, and **40a** with IC_{50} values ranging from 0.24 to 16 μM. These data obtained for compounds **20a**, **21a**, **22a**, and **31a** compared to compounds **15** and **17** tend to show the necessity of a spacer arm between CLB and the sugar carrier. In the set of substances including compounds **20a**, **21a**, **22a**, and **31a** the presence of an aromatic spacer seems to favor the global cytotoxicity slightly (**20a** compared with **21a**, **22a**, and **31a**), whereas the length of the alkyl chain of the spacer does not seem to be the deciding factor, compounds possessing a linker with one, two, or three carbons (respectively, **21a**, **22a**, and **31a**) having comparable cytotoxicities. Concerning the cell line, no real sensitivity or resistance was evident for this set of compounds which have almost the same cytotoxicity on all cell lines used. Some differences in the pattern of cytotoxicity were observed with compounds **15a**, **17a**, and **37a**, IC_{50} values are on the order of 27 μM on MCF7 and M4Beu lines and generally smaller on the other lines (except for compound **37a**). This pattern is comparable to CLB with IC_{50} values upper 100 μM on MCF7 and M4Beu lines. Thereby, the use of amidic bonds via the spacer linkage between the carrier and CLB modified the cytotoxic pattern of the drug by erasing the differences of sensitivity and improving activity on all cell lines. For compound **37a**, a relative resistance of normal diploid human fibroblasts can be observed (IC_{50} > 50 μM) and could be a useful property for a differential effect between tumor and these healthy cells. Such a resistance is also observed for compound **40a** but to a lesser extent. Unlike the other compounds, the two last ones are *O*-glycosides. However, this resistance of fibroblasts could not be considered as a discriminating criterion or a proof of selectivity between malignant and healthy cells since fibroblasts are not surrogates of all normal cells and these cytotoxic activities are obtained in vitro.

All peracetylated compounds were shown to be much more cytotoxic than CLB in vitro. This improvement in potency could be correlated with a retention of the conjugate in the tumor cells through a trapping mechanism similar to that of FDG. The accumulation of FDG in cancer cells[56] is well exploited in PET technology with ^{18}F-FDG and is assigned to metabolic trapping. This preferential assimilation of FDG seems to be controlled by the upregulation of glucose transporters, resulting in accelerated influx in conjunction with higher intracellular levels and efficacy of hexokinase bound to tumor mitochondria, leading to trapping and lower intracellular levels of phosphatase responsible for intracellular retention.[57,58] According to this pathway, the FDG–CLB conjugate may be trapped inside the tumor cells and so be more potent than CLB. Further developments in vivo will, therefore, include the assessment of acute toxicity and antitumor activity in tumor-bearing mice, which is currently made and the demonstration of the affinity of the conjugates for the neoplastic tissues. To perform pharmacokinetic studies and biodistribution in mice in order to prove the preferential targeting to the tumors, the compound must be ^{14}C radiolabeled, and this radiolabeling is currently in progress.

2.2.2. Cellular stress. Most of the alkylating agents, and nitrogen mustards in particular, are known to induce reduced-glutathione (GSH) depletion and ROS production in cells, at least in vitro.[59–62] This resulting oxidative stress is implicated in toxic action on normal cells, especially on respiratory cells[61] or hepatocytes[59] and could be partly responsible for adaptation and resistance of tumor cells to DNA-crosslinking agents.[62] We therefore selected our drug candidates taking into consideration their oxidative stress potency, which should be minimal. To this end, we investigated both parameters in the clonal murine L929 fibroblastic cell line after a 4 h treatment with the cytotoxic compounds in the series, including CLB (Table 2). In parallel, we carried out the calcein-AM assay to ascertain a specific effect on GSH and not an indirect drop due to cytolysis or plasmic membrane impairment. In accordance with previous results reported in the literature,[62] we observed a dramatic fall in GSH cellular content concomitant with ROS production after CLB treatment from 6.25 μM, a non-cytolytic concentration. In the set of new compounds tested here, a similar decrease in GSH at low concentration was observed only with **37a**. In this last case, in contrast to the CLB situation, no ROS production was highlighted. **31a** and **20a** also showed a significant decrease, but at higher concentration (50 μM) and concomitant to a cytolysis effect with the latter. Surprisingly, **22a**, and to a lesser extent **21a** and **17a**, induced a seemingly direct production of ROS in cells. Investigation of the mechanism involved in these cellular responses lies outside the scope of this work, but a

Table 2. Cellular modifications induced by active new synthesized compounds on L929 murine cells

Compound	Growth inhibition at 48 h	ROS production[1]	GSH depletion[2]	Cytolysis[3]
17a	40%	+++	—	—
20a	50%	—	+	+
21a	70%	++	—	—
22a	50%	++++	—	—
31a	50%	—	+	—
37a	ND	—	++++	—
40a	40%	—	—	—
CLB	30%	++++	++++	—

Each compound was tested at four concentrations (6.25, 12.5, 25, 50 μM) for 4 h and cellular response was then assayed as described in Materials and Methods. Positive response is defined as [1]dichlorofluorescein production $\geqslant$ 150% and [2]GS-bimane or [3]calcein production $\leqslant$ 80%. These results are representative of two independent experiments. ++++:positive response at 6.25 μM, +++ at 12.5 μM, ++ at 25 μM, + at 50 μM.

specific mechanism seems to be involved, since **21a**, **22a**, and **31a**, which differ only in the length of the carbon spacer, had different patterns. Finally, according to their cytotoxicity on normal or tumor cells and to their moderate ability or inability to induce oxidative stress, we chose **20a**, **21a**, **31a**, and **40a** to investigate their pharmacodynamic properties in an animal context.

To summarize, the synthesis of new carbohydrate-coupled CLB derivatives with FDG as the sugar-moiety is reported. These studies demonstrate a significant improvement in the in vitro cytotoxicity of peracetylated glucoconjugates compared with the free substance. On the basis of these promising data, the next step will be to evaluate the acute toxicity and in vivo antitumor activity of the selected compounds on animals bearing tumors and to investigate in vivo the targeting of these new agents to tumor tissue.

3. Experimental

3.1. Chemistry

3.1.1. General. All solvents and reagents obtained from commercial sources were used without further purification. Nuclear magnetic resonance spectra (^{1}H NMR and ^{13}C NMR) were performed on a Bruker AM 200 (4.5 T) spectrometer. Chemical shifts are reported in parts per million relative to the internal tetramethylsilane standard for ^{1}H NMR and the solvent for ^{13}C NMR (acetone-d, δ 29.8 ppm; DMSO-d_6, δ 39.5 ppm; $CDCl_3$, δ = 77.2 ppm) The abbreviations used for signal patterns are: br, broad; s, singlet; d, doublet; dd, doublet of doublets; ddd, doublet of doublet of doublets; t, triplet; dt, doublet of triplets; q, quadruplet; qt, quintuplet; m, multiplet. Coupling constants (J values) are in Hertz. Infrared (IR) spectra were recorded on an FTIR-Nicolet Impact 410 spectrophotometer. Melting points were determined on an electrothermal digital apparatus (Reichert) and are uncorrected. Medium-pressure column chromatography was carried out on silica gel 60 (Chromagel, 35–70 μm, SDS) using the indicated solvent mixture expressed as volume/volume ratios. Analytical thin-layer chromatography (TLC) was conducted on precoated silica gel plates (SDS, 60 F_{254}, 0.2 mm thick) with detection by ultraviolet light and (or) visualization with vanillin in sulfuric acid. Mass spectra were recorded on a Bruker Esquire-LC spectrometer. Electrospray ionization mass spectrometry (ESI-MS) was used in positive mode. Microanalysis was performed by the Central Analysis Service (CNRS, Vernaison, France) for C, H, N, and the results were within ±0.4% of the theoretical values, unless otherwise stated.

3.1.2. 3,4,6-Tri-*O*-acetyl-1-[4-{4-[bis(2-chloroethyl)amino]phenyl}butyrate]-2-deoxy-2-fluoro-α,β-D-glucopyranose (14a). To a solution of 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-α,β-D-glucopyranose **9** (330 mg, 1.07 mmol) in CH_2Cl_2 (50 mL), chlorambucil (490 mg, 1.61 mmol), DCC (376 mg, 1.78 mmol), and DMAP (8.7 mg, 0.071 mmol) were added and stirred at room temperature for 22 h. The solid was removed by filtration, and the filtrate was washed with a 1 N aqueous solution of acetic acid and water, dried over $MgSO_4$, and concentrated under vacuum. The resulting residue was purified by silica gel chromatography, using cyclohexane/ethyl acetate (7:3) as eluent, to yield **14a** (360 mg, 57%) as a syrup (α/β ratio = 25:75): IR (CCl_4 solution) ν 1763, 1366, 1240, 1223, 1075, 1038; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.12 (d, 2H, J_o = 8.7 Hz, $H_{Ar}\alpha$), 7.10 (d, 2H, J_o = 8.7 Hz, $H_{Ar}\beta$), 6.69 (d, 2H, $H_{Ar}\alpha$), 6.67 (d, 2H, $H_{Ar}\beta$), 6.50 (d, 1H, $J_{1,2}$ = 3.9 Hz, H-1α), 5.85 (dd, 1H, $J_{1,2}$ = 8.1 Hz, $J_{1,F}$ = 3.1 Hz, H-1β), 5.60 (td, 1H, $J_{2,3} = J_{3,4}$ = 9.6 Hz, $J_{3,F}$ = 12.1 Hz, H-3α), 5.43 (td, 1H, $J_{2,3} = J_{3,4}$ = 9.2 Hz, $J_{3,F}$ = 14.2 Hz, H-3β), 5.14 (t, 1H, $J_{4,5}$ = 9.8 Hz, H-4α), 5.12 (t, 1H, $J_{4,5}$ = 9.7 Hz, H-4β), 4.70 (ddd, 1H, $J_{2,F}$ = 48.6 Hz, H-2α), 4.49 (td, 1H, $J_{2,F}$ = 51.1 Hz, H-2β), 4.40–4.30 (m, 2H, H-6aα, H-6aβ), 4.16–4.04 (m, 3H, H-6bα, H-6bβ, H-5α), 3.90 (ddd, 1H, H-5β), 3.75–3.64 (m, 16H, $N(CH_2CH_2Cl)_2\alpha\beta$), 2.62 (m, 4H, CH_2Ph αβ), 2.48 (m, 4H, $COCH_2\alpha\beta$), 2.10–1.94 (m, 22H, OAc, $CH_2CH_2CH_2\alpha\beta$); ^{13}C NMR (50 MHz, $CDCl_3$)δ 171.55, 171.38, 170.59, 170.18, 169.94, 169.61, 169.54 (CO αβ); 144.63, 144.59 ($C_{Ar}N\alpha\beta$), 130.31, 130.16 ($C_{Ar}CH_2\alpha\beta$), 129.91, 129.87, 112.35 ($CH_{Ar}\alpha\beta$), 91.30 (C-1β, $J_{1,F}$ = 24.1 Hz), 88.39 (C-2β, $J_{2,F}$ = 190.9 Hz), 88.31 (C-1α, $J_{1,F}$ = 22.2 Hz), 86.40 (C-2α, $J_{2,F}$ = 193.7 Hz), 72.90 (C-5β), 72.87 (C-3β, $J_{3,F}$ = 19.4 Hz), 70.73 (C-3α, $J_{3,F}$ = 19.4 Hz), 69.71 (C-5α), 67.76 (C-4α/β, $J_{4,F}$ = 7.2 Hz), 65.47 (C-4α/β, $J_{4,F}$ = 7.6 Hz), 61.46 (C-6αβ), 53.75 ($CH_2N\alpha\beta$), 40.57 ($CH_2Cl\alpha\beta$), 33.82, 33.31 ($CH_2Ph\alpha\beta$, $COCH_2\alpha\beta$), 26.54, 26.46

($CH_2CH_2CH_2$αβ), 20.78, 20.72, 20.64 (CH_3αβ); MS (ESI) *m/z* 594.24 $[M+1]^+$; Anal. ($C_{26}H_{34}Cl_2FNO_9$) C, H, N.

3.1.3. 1-[4-{4-[Bis(2-chloroethyl)amino]phenyl}butyrate]-2-deoxy-2-fluoro-α,β-D-glucopyranose (14c). To a suspension of 10% palladium on charcoal (1.5 g) in THF/EtOH (12:24 mL) was added the benzylated glycoside **14 b** (1.78 g, 2.41 mmol), followed by ammonium formate (850 mg, 13.5 mmol). The mixture was refluxed for 3.5 h and then allowed to cool to room temperature, filtered on Celite, and concentrated under vacuum. The crude product was purified by silica gel chromatography, eluting with cyclohexane/ethyl acetate (1:9), to yield the debenzylated product **14c** (400 mg, 35%) as a brown oil (α/β ratio = 55:45): IR(NaCl) *v* 3418, 1738, 1078 cm^{-1}; ^1H NMR (200 MHz, acetone-d_6)δ 7.18 (d, 4H, J_o = 8.7 Hz, H_{Ar}αβ), 6.81 (d, 4H, H_{Ar}αβ), 6.41 (d, 1H, $J_{1,2}$ = 3.9 Hz, H-1α), 5.85 (dd, 1H, $J_{1,2}$ = 8.1 Hz, $J_{1,F}$ = 3.1 Hz, H-1β), 5.02 (br s, 1H, OH), 4.90 (br s, 1H, OH), 4.71 (br s, 1H, OH), 4.53 (ddd, 1H, $J_{2,3}$ = 9.4 Hz, $J_{2,F}$ = 48.7 Hz, H-2α), 4.25 (td, 1H, $J_{2,3}$ = 8.5 Hz, $J_{2,F}$ = 52.0 Hz, H-2β), 4.19–3.49 (m, 26H, $N(CH_2CH_2Cl)_2$αβ, H-3αβ, H-4αβ, H-5 αβ, 2H-6αβ), 2.65 (t, 4H, *J* = 7.6 Hz, CH_2Phαβ), 2.55–2.41 (m, 4H, $COCH_2$αβ), 2.00–1.93 (m, 4H, $CH_2CH_2CH_2$αβ); ^{13}C NMR (50 MHz, acetone-d_6)δ 171.86, 171.81 (COαβ), 145.25 (C_{Ar}Nαβ), 130.57 ($C_{Ar}CH_2$αβ), 129.96, 112.70 (CH_{Ar}αβ), 92.02 (C-2β, $J_{2,F}$ = 184.2 Hz), 92.00 (C-1β, $J_{1,F}$ = 21.7 Hz), 89.88 (C-2α, $J_{2,F}$ = 184.5 Hz), 89.47 (C-1α, $J_{1,F}$ = 22.6 Hz), 78.00 (C-5β), 75.43 (C-3β, $J_{3,F}$ = 16.6 Hz), 75.18 (C-5α), 72.51 (C-3α, $J_{3,F}$ = 16.8 Hz), 70.44 (C-4α/β, $J_{4,F}$ = 8.0 Hz), 70.16 (C-4α/β, $J_{4,F}$ = 8.1 Hz), 61.62, 61.57 (C-6αβ), 53.47 (CH_2Nαβ), 41.20 (CH_2Cl αβ), 33.99, 33.92, 33.58, 33.37 (CH_2Phαβ, COCH_2αβ), 27.20, 27.05 ($CH_2CH_2CH_2$αβ); MS (ESI) *m/z* 468.54 $[M+1]^+$; Anal. ($C_{20}H_{28}Cl_2FNO_6$) C, H, N.

3.1.4. *N*-(3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosyl)-4-{4-[bis(2-chloroethyl)amino]phenyl}butanamide (15a). Aminosugar **8** (189 mg, 0.61 mmol) was dissolved in anhydrous DMF (10 mL) and chlorambucil (187 mg, 0.61 mmol), DCC (127 mg, 0.61 mmol), and HOBt (92 mg, 0.67 mmol) were added. The solution was stirred for 20 h at room temperature, filtered, concentrated in vacuo, and purified by silica gel chromatography (cyclohexane/ethyl acetate 6:4) to give **15a** (184 mg, 51%) as a white solid: IR (KBr) *v* 3327, 1750, 1676, 1520, 1244, 1069, 1032; ^1H NMR (200 MHz, $CDCl_3$) δ 7.08 (d, 2H, J_o = 8.6 Hz, H_{Ar}), 6.69 (d, 2H, H_{Ar}), 6.04 (d, 1H, *J* = 9.3 Hz, NH), 5.44–5.28 (m, 2H, H-3, H-1), 5.03 (t, 1H, $J_{3,4} = J_{4,5}$ = 9.7 Hz, H-4), 4.31 (dd, 1H, $J_{5,6a}$ = 4.4 Hz, $J_{6a,6b}$ = 12.5 Hz, H-6a), 4.26 (td, 1H, $J_{2,F}$ = 50.8 Hz, $J_{1,2} = J_{2,3}$ = 9.2 Hz, H-2), 4.06 (dd, 1H, $J_{5,6b}$ = 2.0 Hz, H-6b), 3.86 (ddd, 1H, H_5), 3.76–3.58 (m, 8H, $N(CH_2CH_2Cl)_2$), 2.58 (t, 2H, *J* = 7.3 Hz, CH_2Ph), 2.25 (t, 2H, *J* = 7.3 Hz, $COCH_2$), 2.08, 2.07, 2.04 (each s, 3 × 3H, OAc), 1.94 (m, 2H, $CH_2CH_2CH_2$); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.24, 170.68, 169.92, 169.85 (CO), 144.13 (C_{Ar}N), 131.00 ($C_{Ar}CH_2$), 129.88, 112.70 (CH_{Ar}), 88.50 (C-2, $J_{2,F}$ = 191.2 Hz), 77.62 (C-1), 73.78 (C-5), 73.50 (C-3, $J_{3,F}$ = 19.3 Hz), 67.93 (C-4, $J_{4,F}$ = 7.0 Hz), 61.65 (C-6), 53.89 (CH_2N), 40.45 (CH_2Cl), 35.68 ($COCH_2$), 33.89 (CH_2Ph), 26.77 ($CH_2CH_2CH_2$), 20.82, 20.76, 20.70 (CH_3); MS (ESI) *m/z* 593.30 $[M+1]^+$; Anal. ($C_{26}H_{35}Cl_2FN_2O_8$) C, H, N.

3.1.5. 4-{4-[Bis(2-chloroethyl)amino]phenyl}-*N*-(2-deoxy-2-fluoro-β-D-glucopyranosyl) butanamide (15b). To a stirred solution of acetylated compound **15a** (140 mg, 0.24 mmol) in methanol (7 mL) was added sodium methoxide (1 mg). After 4 h at room temperature, the mixture was evaporated to dryness and purified on silica gel (10:90 to 0:100 cyclohexane/ethyl acetate gradient) to afford **15b** (63 mg, 57%) as a solid: mp 168 °C; IR (KBr) *v* 3310, 1655, 1519, 1092, 1057; ^1H NMR (200 MHz, acetone-d_6) δ 7.81 (d, 1H, *J* = 9.1 Hz, NH), 7.06 (d, 2H, J_o = 8.8 Hz, H_{Ar}), 6.70 (d, 2H, H_{Ar}), 5.19 (td, 1H, $J_{1,2} = J_{1,NH}$ = 9.2 Hz, $J_{1,F}$ = 2.3 Hz, H-1), 4.77 (d, 1H, *J* = 4.4 Hz, OH), 4.39 (br s, 1H, OH), 4.06 (td, 1H, $J_{2,3}$ = 9.0 Hz, $J_{2,F}$ = 50.9 Hz, H-2), 3.80–3.58 (m, 12H, $N(CH_2CH_2Cl)_2$, 2H-6, H-3, H-4), 3.63–3.32 (m, 2H, OH, H-5), 2.51 (t, 2H, *J* = 7.6 Hz, CH_2Ph), 2.21 (t, 2H, *J* = 7.9 Hz, $COCH_2$), 1.84 (qt, 2H, $CH_2CH_2CH_2$); ^{13}C NMR (50 MHz, acetone-d_6) δ 173.47 (NHCO), 145.55 (C_{Ar}N), 131.43 ($C_{Ar}CH_2$), 130.36, 113.07 (CH_{Ar}), 92.42 (C-2, $J_{2,F}$ = 183.6 Hz), 79.10 (C-5), 78.06 (C-1, $J_{1,F}$ = 23.2 Hz), 76.76 (C-3, $J_{3,F}$ = 16.8 Hz), 71.51 (C-4, $J_{4,F}$ = 7.7 Hz), 62.47 (C-6), 53.89 (CH_2N), 41.62 (CH_2Cl), 36.02 ($COCH_2$), 34.70 (CH_2Ph), 28.07 ($CH_2CH_2CH_2$); MS (ESI) *m/z* 467.15 $[M+1]^+$; Anal. ($C_{20}H_{29}Cl_2FN_2O_5$) C, H, N.

3.1.6. *N*-(3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosyl)-*N*′-{3-(4-[bis(2-chloroethyl)amino]phenyl)propyl}-urea (17a). To aminosugar **8** (200 mg, 0.65 mmol) dissolved in CH_2Cl_2/THF (1.5:2 mL) was added isocyanate **16** (215 mg, 0.72 mmol) in CH_2Cl_2 (0.5 mL), and the mixture was stirred overnight at room temperature. After evaporation of the solvent, the concentrate was purified by column chromatography on silica gel with ethyl acetate/petroleum benzine (6:4 to 7:3 gradient) as developing solvent to give compound **17a** (200 mg, 50%) as a red powder: mp 91 °C; IR (NaCl) *v* 1746, 1364, 1230, 1029; ^1H NMR (200 MHz, $CDCl_3$) δ 7.06 (d, 2H, J_o = 8.6 Hz, H_{Ar}), 6.63 (d, 2H, H_{Ar}), 5.46–5.23 (m, 2H, H-1, H-3), 5.01 (t, 1H, $J_{3,4} = J_{4,5}$ = 9.7 Hz, H-4), 4.84 (br t, 1H, *J* = 5.1 Hz, N*H*CH_2), 4.33 (dd, 1H, $J_{6a,6b}$ = 12.5 Hz, $J_{5,6a}$ = 4.3 Hz, H-6a), 4.20 (td, 1H, $J_{2,F}$ = 50.5 Hz, $J_{1,2} = J_{2,3}$ = 9.2 Hz, H-2), 4.06 (dd, 1H, $J_{5,6b}$ = 2.1 Hz, H-6b), 3.82 (ddd, 1H, H-5), 3.65 (m, 8H, $N(CH_2CH_2Cl)_2$), 3.23 (m, 2H, $NHCH_2$), 2.55 (t, 2H, *J* = 7.4 Hz, CH_2Ph), 2.09, 2.07, 2.05 (each s, 3 × 3H, OAc), 1.78 (qt, 2H, $CH_2CH_2CH_2$); ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.76, 170.03, 169.91 ($COCH_3$), 156.84 (NH*C*O), 144.43 (C_{Ar}N), 130.53 ($C_{Ar}CH_2$), 129.63, 112.29 (CH_{Ar}), 88.54 (C-2, $J_{2,F}$ = 190.6 Hz), 79.59 (C-1, $J_{1,F}$ = 22.2 Hz), 73.67 (C-3, $J_{3,F}$ = 19.5 Hz), 73.20 (C-5), 68.15 (C-4, $J_{4,F}$ = 7.1 Hz), 61.72 (C-6), 53.63 (CH_2N), 40.61 (CH_2Cl), 40.03 ($NHCH_2$), 31.99, 31.87 (CH_2Ph, $CH_2CH_2CH_2$), 20.73 (CH_3); MS (ESI) *m/z* 608.19 $[M+1]^+$; Anal. ($C_{26}H_{36}Cl_2FN_3O_8$) C, H, N.

3.1.7. *N*-{3-(4-[Bis(2-chloroethyl)amino]phenyl)propyl}-*N*′-(2-deoxy-2-fluoro-β-D-glucopyranosyl)urea (17b). To a stirred solution of acetylated compound **17a**

(200 mg, 0.33 mmol) in methanol (12 mL) was added sodium methoxide (2 mg), and the mixture was stirred at room temperature for 3 h. After neutralization with IRC 50 Amberlite ion-exchange resin (H^+), filtration and evaporation to dryness, the residue was purified on silica gel (10% methanol in ethyl acetate) to yield compound **17b** (75 mg, 45%) as an oil: IR (KBr) ν 3348, 1750, 1078, 1028; ^{1}H NMR (200 MHz, acetone-d_6) δ 7.09 (d, 2H, J_o = 8.8 Hz, H_{Ar}), 6.72 (d, 2H, H_{Ar}), 6.34 (d, 1H, J = 9.5 Hz, C_1NHCO), 5.80 (t, 1H, J = 5.8 Hz, N*H*CH_2), 5.09 (td, 1H, $J_{1,2}$ = 9.3 Hz, $J_{1,F}$ = 2.0 Hz, H-1), 4.80 (d, 1H, J = 4.4 Hz, OH), 4.43 (d, 1H, J = 4.4 Hz, OH), 4.02 (td, 1H, $J_{2,F}$ = 50.9 Hz, $J_{2,3}$ = 8.9 Hz, H-2), 3.84–3.67 (m, 12H, N(CH_2CH_2Cl)$_2$, H-3, H-4, 2H-6), 3.40–3.36 (m, 2H, H-5, OH), 3.16 (t, 2H, J = 6.4 Hz, NHC*H*$_2$), 2.53 (t, 2H, J = 7.7 Hz, C*H*$_2$Ph), 1.74 (qt, 2H, $CH_2CH_2CH_2$); ^{13}C NMR (50 MHz, acetone-d_6) δ 158.49 (NHCO), 145.38 ($C_{Ar}N$), 131.37 ($C_{Ar}CH_2$), 130.23, 113.01 (CH_{Ar}), 92.30 (C-2, $J_{2,F}$ = 195.0 Hz), 79.97 (C-1, $J_{1,F}$ = 25.0 Hz), 78.50 (C-5), 76.73 (C-3, $J_{3,F}$ = 16.9 Hz), 71.39 (C-4, $J_{4,F}$ = 7.2 Hz), 62.36 (C-6), 53.84 (CH_2N), 41.62 (CH_2Cl), 40.27 ($NHCH_2$), 32.84, 32.55 (*C*H_2Ph, $CH_2CH_2CH_2$); MS (ESI) *m/z* 482.16 $[M+1]^+$; Anal. ($C_{20}H_{30}Cl_2FN_3O_5$) C, H, N.

3.1.8. *N*-(3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosyl)-4-(4-{4-[bis(2-chloroethyl)amino]phenyl}butanamido)benzamide (20a). Compound **20a** was prepared according to the procedure described for compound **18c**, starting from chlorambucil (217 mg, 0.72 mmol) and amine **19a** (277 mg, 0.65 mmol). The crude product was purified by column chromatography using petroleum benzine/ethyl acetate (5:5 to 3:7) as the eluent, to give compound **20a** (370 mg, 80%) as a beige powder: mp 165 °C; IR (KBr) ν 3325, 1749, 1670, 1520, 1365, 1244, 1066, 1036; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.80 (d, 2H, J_o = 8.6 Hz, H_{Ar}), 7.66 (s, 1H, PhN*H*CO), 7.59 (d, 2H, J_o, H_{Ar}), 7.39 (d, 1H, J = 9.1 Hz, C_1NH), 7.07 (d, 2H, J'_o = 8.6 Hz, H_{Ar}), 6.63 (d, 2H, J'_o, H_{Ar}), 5.58 (br t, 1H, $J_{1,2}$ = 9.0 Hz, H-1), 5.40 (td, 1H, $J_{2,3}$ = $J_{3,4}$ = 9.2 Hz, $J_{3,F}$ = 13.8 Hz, H-3), 5.06 (t, 1H, $J_{4,5}$ = 9.8 Hz, H-4), 4.55 (td, 1H, $J_{2,F}$ = 50.3 Hz, H-2), 4.32 (dd, 1H, $J_{5,6a}$ = 4.0 Hz, $J_{6a,6b}$ = 12.4 Hz, H-6a), 4.06 (dd, 1H, $J_{5,6b}$ = 1.5 Hz, H-6b), 3.86 (ddd, 1H, H-5), 3.73–3.55 (m, 8H, N(CH_2CH_2Cl)$_2$), 2.61 (t, 2H, J = 7.3 Hz, C*H*$_2$Ph), 2.38 (t, 2H, J = 7.0 Hz, $COCH_2$), 2.07–1.73 (m, 11H, $CH_2CH_2CH_2$, 3OAc); ^{13}C NMR (50 MHz, MeOD) δ 174.73, 172.27, 171.47, 171.34, 169.88 (CO), 145.99 ($C_{Ar}N$), 143.91 ($C_{Ar}NH$), 131.60 ($C_{Ar}CH_2$), 130.66, 129.66 (CH_{Ar}), 129.30 ($C_{Ar}CO$), 120.23, 113.54 (CH_{Ar}), 89.46 (C-2, $J_{2,F}$ = 187.7 Hz), 79.24 (C-1, $J_{1,F}$ = 23.2 Hz), 74.95 (C-3, $J_{3,F}$ = 19.4 Hz), 74.60 (C-5), 69.55 (C-4, $J_{4,F}$ = 7.3 Hz), 63.03 (C-6), 54.53 (CH_2N), 41.67 (CH_2Cl), 37.41 ($COCH_2$), 35.20 (*C*H_2Ph), 28.50 ($CH_2CH_2CH_2$), 20.58 (CH_3); MS (ESI) *m/z* 712.33 $[M+1]^+$; Anal. ($C_{33}H_{40}Cl_2FN_3O_9$) C, H, N.

3.1.9. 4-(4-{4-[Bis(2-chloroethyl)amino]phenyl}butanamido)-*N*-(2-deoxy-2-fluoro-β-D-glucopyranosyl)benzamide (20b). Zemplèn deacetylation as for **17b** and chromatography on silica gel (5% methanol in ethyl acetate) afforded compound **20b** (105 mg, 74%) as a white powder: decomposition at 230 °C; IR (KBr) ν 3301, 1658, 1519, 1089, 1045; ^{1}H NMR (200 MHz, DSMO) δ 10.12 (s, 1H, PhN*H*CO), 9.04 (d, 1H, J = 8.9 Hz, C_1NH), 7.83 (d, 2H, J = 8.8 Hz, H_{Ar}), 7.67 (d, 2H, J_o, H_{Ar}), 7.02 (d, 2H, J'_o = 8.6 Hz, H_{Ar}), 6.64 (d, 2H, J'_o, H_{Ar}), 5.54 (d, 1H, J = 5.4 Hz, OH), 5.19 (m, 2H, OH, H-1), 4.59 (t, 1H, J = 5.6 Hz, OH), 4.29 (td, 1H, $J_{1,2}$ = $J_{2,3}$ = 8.9 Hz, $J_{2,F}$ = 50.6 Hz, H-2), 3.70–3.05 (m, 13H, N(CH_2CH_2Cl)$_2$, H-3, H-4, H-5, 2H-6), 2.48 (m, 2H, C*H*$_2$Ph), 2.31 (t, 2H, J = 7.3 Hz, $COCH_2$), 1.81 (qt, 2H, $CH_2CH_2CH_2$); ^{13}C NMR (50 MHz, DMSO-d_6) δ 171.54, 166.02 (CO), 144.44 ($C_{Ar}N$), 142.47 ($C_{Ar}NH$), 129.72 ($C_{Ar}CH_2$), 129.32, 128.40 (CH_{Ar}), 127.56 ($C_{Ar}CO$), 118.15, 111.91 (CH_{Ar}), 91.10 (C-2, $J_{2,F}$ = 182.2 Hz), 78.73 (C-5), 77.42 (C-1, $J_{1,F}$ = 22.9 Hz), 75.18 (C-3, $J_{3,F}$ = 16.2 Hz), 69.85 (C-4, $J_{4,F}$ = 7.6 Hz), 60.55 (C-6), 52.22 (CH_2N), 41.14 (CH_2Cl), 35.85 ($COCH_2$), 33.54 (*C*H_2Ph), 26.85 ($CH_2CH_2CH_2$); MS (ESI) *m/z* 586.14 $[M+1]^+$; Anal. ($C_{27}H_{34}Cl_2FN_3O_6$) C, H, N.

3.1.10. *N*-[2-(3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosylamino)-2-oxoethyl]-4-{4-[bis(2-chloroethyl)-amino]phenyl} butanamide (21a). Compound **21a** was prepared according to the procedure described for compound **15a**, starting from amino derivative **19b** (1.02 g, 2.80 mmol) and chlorambucil (939 mg, 3.09 mmol). Purification on silica gel (4:6 to 2:8 petroleum benzine/ethyl acetate gradient) gave compound **21a** (945 mg, 64%) as a white solid: mp 106 °C; IR (KBr) ν 3368, 1750, 1653, 1519, 1230, 1069, 1034; ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.08 (d, 1H, J = 9.1 Hz, C_1NH), 7.06 (d, 2H, J_o = 8.5 Hz, H_{Ar}), 6.63 (d, 2H, H_{Ar}), 6.46 (br t, 1H, J = 4.6 Hz, CH_2NH), 5.41–5.29 (m, 2H, H-1, H-3), 5.03 (t, 1H, $J_{3,4}$ = $J_{4,5}$ = 9.7 Hz, H-4), 4.40 (td, 1H, $J_{1,2}$ = $J_{2,3}$ = 9.0 Hz, $J_{2,F}$ = 50.3 Hz, H-2), 4.26 (dd, 1H, $J_{5,6a}$ = 4.2 Hz, $J_{6a,6b}$ = 12.7 Hz, H-6a), 4.09–4.01 (m, 3H, H-6b, $COCH_2NH$), 3.84 (ddd, 1H, $J_{5,6b}$ = 1.7 Hz, H-5), 3.75–3.57 (m, 8H, N(CH_2CH_2Cl)$_2$), 2.56 (t, 2H, J = 7.3 Hz, C*H*$_2$Ph), 2.26 (t, 2H, J = 7.3 Hz, $COCH_2$), 2.08, 2.04, 1.99 (each s, 3 × 3H, OAc), 1.92 (qt, 2H, $CH_2CH_2CH_2$); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.98, 170.71, 170.01, 169.84, 169.74 (CO), 145.58 ($C_{Ar}N$), 130.36 ($C_{Ar}CH_2$), 129.78, 112.36 (CH_{Ar}), 88.37 (C-2, $J_{2,F}$ = 191.0 Hz), 77.38 (C-1), 73.76–73.29 (C-3, C-5), 68.02 (C-4, $J_{4,F}$ = 6.7 Hz), 61.77 (C-6), 53.70 (CH_2N), 43.68 ($COCH_2NH$), 40.67 (CH_2Cl), 35.50 ($COCH_2CH_2$), 34.02 (*C*H_2Ph), 27.19 ($CH_2CH_2CH_2$), 20.79, 20.74 (CH_3); MS (ESI) *m/z* 650.26 $[M+1]^+$; Anal. ($C_{28}H_{38}Cl_2FN_3O_9$) C, H, N.

3.1.11. 4-{4-[Bis(2-chloroethyl)amino]phenyl}-*N*-[2-(2-deoxy-2-fluoro-β-D-glucopyranosylamino)-2-oxoethyl]-butanamide (21b). Zemplèn deacetylation as for **17b** and chromatography on silica gel (10% methanol in ethyl acetate) gave compound **21b** (100 mg, 98%) as a white powder: mp 110 °C; IR (KBr) ν 3315, 1647, 1519, 1249, 1081, 1039; ^{1}H NMR (200 MHz, acetone–d_6) δ 8.19 (d, 1H, J = 9.3 Hz, C_1NH), 7.51 (t, 1H, J = 5.5 Hz, CH_2NH), 7.08 (d, 2H, J_o = 8.6 Hz, H_{Ar}), 6.70 (d, 2H, H_{Ar}), 5.21 (td, 1H, $J_{1,2}$ = 9.1 Hz, $J_{1,F}$ = 1.8 Hz, H-1), 4.99 (br d, 1H, J = 3.4 Hz, OH), 4.62 (br s, 1H, OH), 4.15 (td, 1H, $J_{2,3}$ = 8.8 Hz,

$J_{2,F}$ = 50.8 Hz, H-2), 4.09 (br s, 1H, OH), 3.95 (d, 2H, COCH_2NH), 3.79–3.66 (m, 11H, N(CH_2CH_2Cl)$_2$, H-3, 2H-6), 3.42 (m, 2H, H-4, H-5), 2.53 (t, 2H, J = 7.5 Hz, CH_2Ph), 2.27 (t, 2H, J = 7.3 Hz, COCH_2CH_2), 1.86 (qt, 2H, $CH_2CH_2CH_2$); ^{13}C NMR (50 MHz, acetone-d_6) δ 174.39, 171.08 (CO), 145.45 (C_{Ar}N), 131.49 ($C_{Ar}CH_2$), 130.36, 113.04 (CH_{Ar}), 92.13 (C-2, $J_{2,F}$ = 183.9 Hz), 79.08 (C-5), 78.13 (C-1, $J_{1,F}$ = 23.3 Hz), 76.55 (C-3, $J_{3,F}$ = 16.7 Hz), 70.94 (C-4, $J_{4,F}$ = 4.0 Hz), 62.02 (C-6), 53.89 (CH_2N), 43.31 (COCH_2NH), 41.65 (CH_2Cl), 35.87 (COCH_2CH_2), 34.76 (CH_2Ph), 28.27 ($CH_2CH_2CH_2$); MS (ESI) m/z 524.12 [M+1]$^+$; Anal. ($C_{22}H_{32}Cl_2FN_3O_6$) C, H, N.

3.1.12. *N*-[4-(3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosylamino)-4-oxobutyl]-4-{4-[bis(2-chloroethyl)-amino]phenyl}butanamide (22a). Compound **22a** was prepared according to the procedure described for compound **18c**, starting from chlorambucil (130 mg, 0.43 mmol) and amine **19c** (168 mg, 0.43 mmol). The crude product was purified by column chromatography using ethyl acetate as developing solvent, to give compound **22a** (70 mg, 24%) as a beige powder: mp 95 °C; IR (NaCl) ν 3295, 1749, 1647, 1542, 1366, 1230, 1069, 1034; ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.26 (d, 1H, J = 9.0 Hz, C_1NH), 7.07 (d, 2H, J_o = 8.6 Hz, H_{Ar}), 6.63 (d, 2H, H_{Ar}), 5.86 (br t, 1H, J = 6.0 Hz, CH_2NHCO), 5.45–5.34 (m, 2H, H-1, H-3), 5.06 (t, 1H, $J_{3,4}$ = $J_{4,5}$ = 9.8 Hz, H-4), 4.47 (td, 1H, $J_{1,2}$ = $J_{2,3}$ = 9.1 Hz, $J_{2,F}$ = 50.3 Hz, H-2), 4.29 (dd, 1H, $J_{6a,6b}$ = 12.5 Hz, $J_{5,6a}$ = 4.3 Hz, H-6a), 4.08 (dd, 1H, $J_{5,6b}$ = 2.1 Hz, H-6b), 3.83 (ddd, 1H, H-5), 3.76–3.58 (m, 8H, N(CH_2CH_2Cl)$_2$), 3.43 (m, 1H, CHNH), 3.17 (m, 1H, CH'NH), 2.56 (t, 2H, J = 7.3 Hz, CH_2Ph), 2.30–2.17 (m, 4H, 2CH_2CO), 2.07, 2.05, 2.04 (each s, 3 × 3H, OAc), 2.00–1.76 (m, 4H, 2$CH_2CH_2CH_2$); ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.45, 173.62 (NHCO), 170.76, 170.04, 169.75 (COCH$_3$), 144.52 (C_{Ar}N), 130.48 ($C_{Ar}CH_2$), 129.78, 112.32 (CH_{Ar}), 88.42 (C-2, $J_{2,F}$ = 189.8 Hz), 77.63 (C-1, $J_{1,F}$ = 23.2 Hz), 73.65 (C-5), 73.64 (C-3, $J_{3,F}$ = 22.6 Hz), 68.06 (C-4, $J_{4,F}$ = 6.7 Hz), 61.82 (C-6), 53.69 (CH_2N), 40.65 (CH_2Cl), 38.42 ($CH_2CH_2CH_2$NH), 36.10 ($CH_2CH_2CH_2$Ph), 34.19 ($CH_2CH_2CH_2$Ph), 33.59 ($CH_2CH_2CH_2$NH), 27.56 ($CH_2CH_2CH_2$Ph), 26.42 ($CH_2CH_2CH_2$NH), 20.86, 20.79, 20,71 (CH_3); MS (ESI) m/z 678.29 [M+1]$^+$; Anal. ($C_{30}H_{42}Cl_2FN_3O_9$) C, H, N.

3.1.13. 4-{4-[Bis(2-chloroethyl)amino]phenyl}-*N*-[4-(2-deoxy-2-fluoro-β-D-glucopyranosylamino)-4-oxobutyl]butanamide (22b). Zemplèn deacetylation as for **17b** and chromatography on silica gel (10% methanol in ethyl acetate) gave compound **22b** (69 mg, 72%) as a solid: mp 161 °C; IR (KBr) ν 3525, 3309, 1641, 1550, 1519, 1355, 1093, 1045; ^{1}H NMR (200 MHz, acetone-d_6) δ 8.13 (d, 1H, J = 9.2 Hz, C_1NH), 7.18–7.05 (m, 3H, H_{Ar}, CH_2NH), 6.72 (d, 2H, J_o = 8.7 Hz, H_{Ar}), 5.19 (td, 1H, $J_{1,2}$ = 9.0 Hz, $J_{1,F}$ = 2.2 Hz, H-1), 4.78 (br s, 1H, OH), 4.45 (br s, 1H, OH), 4.12 (td, 1H, $J_{2,3}$ = 9.0 Hz, $J_{2,F}$ = 50.9 Hz, H-2), 3.90–3.51 (m, 12H, N(CH_2CH_2Cl)$_2$, H-3, H-4, 2H-6), 3.40 (m, 2H, OH, H-5), 3.23 (q, 2H, J = 6.4 Hz, CH_2NH), 2.52 (t, 2H, J = 7.4 Hz, CH_2Ph), 2.28–2.13 (m, 4H, 2CH_2CO), 1.96–1.73 (m, 4H, 2$CH_2CH_2CH_2$); ^{13}C NMR (50 MHz, MeOD) δ 176.30, 176.18 (NHCO), 145.98 (C_{Ar}N), 131.73 ($C_{Ar}CH_2$), 130.60, 113.54 (CH_{Ar}), 92.27 (C-2, $J_{2,F}$ = 184.4 Hz), 79.75 (C-5), 78.59 (C-1, $J_{1,F}$ = 23.3 Hz), 76.93 (C-3, $J_{3,F}$ = 16.9 Hz), 71.11 (C-4, $J_{4,F}$ = 7.6 Hz), 62.35 (C-6), 54.55 (CH_2N), 41.71 (CH_2Cl), 39.75 ($CH_2CH_2CH_2$NH), 36.55 ($CH_2CH_2CH_2$Ph), 35.23 ($CH_2CH_2CH_2$Ph), 34.34 ($CH_2CH_2CH_2$NH), 28.94 ($CH_2CH_2CH_2$Ph), 26.34 ($CH_2CH_2CH_2$NH); MS (ESI) m/z 552.14 [M+1]$^+$; Anal. ($C_{24}H_{36}Cl_2FN_3O_6$) C, H, N.

3.1.14. *N*-[3-(3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosylamino)-3-oxopropyl]-4-{4-[bis(2-chloroethyl)amino]phenyl}butanamide (31a). Compound **31a** was prepared according to the procedure described for compound **18c**, starting from acid **30** (33 mg, 0.09 mmol) and aminosugar **8** (24.5 mg, 0.08 mmol). The crude product was purified by silica gel chromatography (2:8 to 1:9 petroleum benzine/ethyl acetate gradient), to give expected compound **31a** (47 mg, 88%) as a beige solid: mp 103 °C; IR (KBr) ν 3309, 1751, 1676, 1647, 1519, 1367, 1228, 1066, 1034; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.90 (d, 1 H, J = 8.9 Hz, C_1NH), 7.04 (d, 2H, J_o = 8.5 Hz, H_{Ar}), 6.61 (d, 2H, H_{Ar}), 6.37 (br t, 1H, J = 5.5 Hz, CH_2NHCO), 5.46–5.30 (m, 2H, H-1, H-3), 5.03 (t, 1H, $J_{3,4}$ = $J_{4,5}$ = 9.8 Hz, H-4), 4.40 (td, 1H, $J_{1,2}$ = $J_{2,3}$ = 9.1 Hz, $J_{2,F}$ = 50.4 Hz, H-2), 4.28 (dd, 1H, $J_{6a,6b}$ = 12.5 Hz, $J_{5,6a}$ = 4.3 Hz, H-6a), 4.04 (dd, 1H, $J_{5,6b}$ = 1.4 Hz, H-6b), 3.84 (m, 1H, H-5), 3.72–3.47 (m, 10H, N(CH_2CH_2Cl)$_2$, CH_2NH), 2.52 (m, 4H, CH_2Ph, COCH_2CH_2NH), 2.19–2.04 (m, 11H, COC$H_2CH_2CH_2$, 3OAc), 1.87 (m, 2H, $CH_2CH_2CH_2$); ^{13}C NMR (50 MHz, MeOD) δ 176.25, 174.45 (CONH), 172.19, 171.43, 171.27 (COCH$_3$), 145.99 (C_{Ar}N), 131.81 ($C_{Ar}CH_2$), 130.63, 113.57 (CH_{Ar}), 89.57 (C-2, $J_{2,F}$ = 188.6 Hz), 78.51 (C-1, $J_{1,F}$ = 22.9 Hz), 74.81 (C-3, $J_{3,F}$ = 19.5 Hz), 74.53 (C-5), 69.50 (C-4, $J_{4,F}$ = 7.3 Hz), 62.98 (C-6), 54.58 (CH_2N), 41.72 (CH_2Cl), 36.44, 35.15 (COCH_2CH_2NH, CO$CH_2CH_2CH_2$Ph), 28.89 ($CH_2CH_2CH_2$), 20.58 (CH_3); MS (ESI) m/z 664.33 [M+1]$^+$; Anal. ($C_{29}H_{40}Cl_2FN_3O_9$) C, H, N.

3.1.15. 4-{4-[Bis(2-chloroethyl)amino]phenyl}-*N*-[3-(2-deoxy-2-fluoro-β-D-glucopyranosylamino)-3-oxopropyl]-butanamide (31b). Zemplèn deacetylation as for **17b** and chromatography on silica gel (10% methanol in ethyl acetate) gave compound **31b** (80 mg, 83%) as a white solid: mp 179 °C; IR (KBr) ν 3525, 3305, 1647, 1544, 1091, 1043; ^{1}H NMR (200 MHz, acetone-d_6) δ 8.05 (d, 1H, J = 9.1 Hz, C_1NH), 7.06 (m, 3H, H_{Ar}, CH_2NHCO), 6.72 (d, 2H, J_o = 8.8 Hz, H_{Ar}), 5.19 (td, 1H, $J_{1,2}$ = 9.1 Hz, $J_{1,F}$ = 2.3 Hz, H-1), 4.84 (d, 1H, J = 4.5 Hz, OH), 4.48 (d, 1H, J = 4.5 Hz, OH), 4.09 (td, 1H, $J_{2,3}$ = 9.0 Hz, $J_{2,F}$ = 50.9 Hz, H-2), 3.80–3.68 (m, 11H, N(CH_2CH_2Cl)$_2$, H-3, 2H-6), 3.45–3.38 (m, 5H, H-4, H-5, CH_2NH, OH), 2.47 (m, 4H, CH_2Ph, COCH_2CH$_2$NH), 2.15 (m, 2H, COC$H_2CH_2CH_2$), 1.83 (m, 2H, $CH_2CH_2CH_2$); ^{13}C NMR (50 MHz, MeOD) δ 176.19, 174.58 (NHCO), 145.92 (C_{Ar}N), 131.73 ($C_{Ar}CH_2$), 130.60, 113.49 (CH_{Ar}), 92.20 (C-2, $J_{2,F}$ = 184.8 Hz), 79.71 (C-5), 78.50 (C-1, $J_{1,F}$ = 23.1 Hz), 76.90 (C-3, $J_{3,F}$ = 16.8 Hz), 71.13 (C-4, $J_{4,F}$ = 7.5 Hz), 62.38 (C-6), 54.53 (CH_2N), 41.69 (CH_2Cl), 36.52,

35.16 ($CO\mathit{C}H_2\mathit{C}H_2NH$, $CO\mathit{C}H_2CH_2\mathit{C}H_2Ph$), 28.87 ($CH_2\mathit{C}H_2CH_2$); MS (ESI) *m/z* 538.06 $[M+1]^+$; Anal. ($C_{23}H_{34}Cl_2FN_3O_6$) C, H, N.

3.1.16. *N*-[4-(4-{4-[Bis(2-chloroethyl)amino]phenyl}butanoyloxy)phenyl]-2-deoxy-2-fluoro-β-D-glucopyranosylamine (34b). A solution of the benzylated derivative **34a** (189 mg, 0.23 mmol) in EtOH/THF (5:2.5 mL) was stirred under hydrogen atmosphere in the presence of 10% palladium on charcoal (47 mg) for 18 h at room temperature. After filtration of the catalyst on Celite and evaporation of the solvent, the crude product was purified by silica gel chromatography (cyclohexane/ethyl acetate 1:9) to yield the deprotected product **34b** (100 mg, 78%) as a white powder (α/β ratio =20:80): IR (KBr) *ν* 3349, 1750, 1519, 1201, 1179, 1135, 1078, 1012; ^{1}H NMR (200 MHz, acetone-d_6) *δ* 7.10 (d, 2H, J_o = 8.5 Hz, H_{Ar}), 6.86 (d, 2H, J'_o = 9.1 Hz, H_{Ar}), 6.75 (2d, 4H, H_{Ar}), 6.00 (d, 1H, $J_{1,NH}$ = 8.4 Hz, NHβ), 5.76 (d, 1H, $J_{1,NH}$ = 4.8 Hz, NHα), 5.27 (br t, 1H, $J_{1,2}$ = 5.2 Hz, H-1α), 4.83 (br t, 1H, $J_{1,2}$ = 8.5 Hz, H-1β), 4.75 (d, 1H, *J* = 4.5 Hz, OHβ), 4.61 (d, 1H, *J* = 4.4 Hz, OHα), 4.41 (d, 1H, *J* = 4.0 Hz, OHβ), 4.38 (d, 1H, *J* = 5.0 Hz, OHα), 4.13 (td, 1H, $J_{2,3}$ = 8.7 Hz, $J_{2,F}$ = 51.8 Hz, H-2β), 4.01 (m, 1H, H-3β), 3.82–3.38 (m, 13H, $N(CH_2CH_2Cl)_2$, H-4, H-5, 2H-6, OH), 2.60 (t, 2H, *J* = 7.5 Hz, $C\mathit{H}_2Ph$), 2.50 (t, 2H, *J* = 7.3 Hz, $COCH_2$), 1.94 (qt, 2H, *J* = 7.4 Hz, $CH_2\mathit{C}H_2CH_2$); ^{13}C NMR (50 MHz, acetone-d_6) 172.74 (CO), 145.65, 145.00 ($2C_{Ar}N$), 143.91 ($C_{Ar}O$), 130.98 ($C_{Ar}CH_2$), 130.41, 122.94, 114.84, 113.06 (CH_{Ar}), 92.84 (C-2, $J_{2,F}$ = 184.7 Hz), 83.67 (C-1, $J_{1,F}$ = 20.2 Hz), 77.92 (C-5), 77.01 (C-3, $J_{3,F}$ = 16.9 Hz), 71.73 (C-4, $J_{4,F}$ = 7.7 Hz), 62.53 (C-6), 53.86 (NCH_2), 41.59 (CH_2Cl), 34.54, 33.93 ($\mathit{C}H_2Ph$, $CO\mathit{C}H_2$), 27.71 ($CH_2\mathit{C}H_2CH_2$); MS (ESI) *m/z* 559.10 $[M+1]^+$; Anal. ($C_{26}H_{33}Cl_2FN_2O_6$) C, H, N.

3.1.17. *N*-[4-(3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosyloxy)phenyl]-4-{4-[bis(2-chloroethyl)amino]-phenyl}butanamide (37a). Compound **37a** was prepared according to the procedure described for compound **15a**, starting from amino derivative **36** (318 mg, 0.80 mmol) and chlorambucil (243 g, 0.80 mmol). Purification on silica gel (cyclohexane/ethyl acetate 6:4) gave compound **37a** (431 mg, 79%) as a white solid: mp 110–113 °C; IR (KBr) *ν* 3326, 1757, 1671, 1519, 1221, 1069, 1044; ^{1}H NMR (200 MHz, $CDCl_3$) *δ* 7.44 (d, 2H, J_o = 9.0 Hz, H_{Ar}), 7.23 (br s, 1H, NH), 7.09 (d, 2H, J'_o = 8.6 Hz, H_{Ar}), 7.02 (d, 2H, J_o, H_{Ar}), 6.63 (d, 2H, J'_o, H_{Ar}), 5.41 (td, 1H, $J_{3,4}$ = $J_{2,3}$ = 9.2 Hz, $J_{3,F}$ = 14.5 Hz, H-3), 5.10 (t, 1H, $J_{4,5}$ = 9.5 Hz, H-4), 5.07 (dd, 1H, $J_{1,F}$ = 2.7 Hz, $J_{1,2}$ = 7.5 Hz, H-1), 4.56 (ddd, 1H, $J_{2,F}$ = 50.4 Hz, H-2), 4.30 (dd, 1H, $J_{5,6a}$ = 5.3 Hz, $J_{6a,6b}$ = 12.3 Hz, H-6a), 4.15 (dd, 1H, $J_{5,6b}$ = 2.1 Hz, H-6b), 3.79 (ddd, 1H, H-5), 3.73–3.58 (m, 8H, $N(CH_2CH_2Cl)_2$), 2.62 (t, 2H, *J* = 7.3 Hz, $C\mathit{H}_2Ph$), 2.34 (t, 2H, *J* = 7.3 Hz, $COCH_2$), 2.12, 2.08, 2.05 (each s, 3 × 3H, OAc), 2.11–1.98 (m, 2H, $CH_2C\mathit{H}_2CH_2$); ^{13}C NMR (50 MHz, $CDCl_3$) *δ* 171.22, 170.66, 170.13, 169.65 (CO), 153.16 ($C_{Ar}O$), 144.48 ($C_{Ar}N$), 133.94 ($C_{Ar}NH$), 130.52 ($\mathit{C}_{Ar}CH_2$), 129.83, 121.40, 118.12, 112.26 (CH_{Ar}), 99.39 (C-1, $J_{1,F}$ = 23.2 Hz), 89.09 (C-2, $J_{2,F}$ = 190.6 Hz), 72.78 (C-3, $J_{3,F}$ = 19.9 Hz), 72.10 (C-5), 68.14 (C-4, $J_{4,F}$ = 7.3 Hz), 61.91 (C-6), 53.66 (CH_2N), 40.63 (CH_2Cl), 36.79 ($CO\mathit{C}H_2$), 34.03 ($\mathit{C}H_2Ph$), 27.21 ($CH_2\mathit{C}H_2CH_2$), 20.81, 20.79, 20.68 (CH_3); MS (ESI) *m/z* 685.30 $[M+1]^+$; Anal. ($C_{32}H_{39}Cl_2FN_2O_9$) C, H, N.

3.1.18. 4-{4-[Bis(2-chloroethyl)amino]phenyl}-*N*-[4-(2-deoxy-2-fluoro-β-D-glucopyranosyloxy)phenyl]butanamide (37b). Zemplèn deacetylation as for **17b** and precipitation in diisopropylic ether gave compound **37b** (1.1 g, 98%) as a white solid: mp 170 °C; IR (KBr) *ν* 3293, 1667, 1521, 1221, 1073, 1049; ^{1}H NMR (200 MHz, acetone-d_6) *δ* 9.07 (s, 1H, NH), 7.61 (d, 2H, J_o = 9.0 Hz, H_{Ar}), 7.10 (d, 2H, J'_o = 8.7 Hz, H_{Ar}), 7.03 (d, 2H, J_o, H_{Ar}), 6.73 (d, 2H, J'_o, H_{Ar}), 5.19 (dd, 1H, $J_{1,2}$ = 7.7 Hz, $J_{1,F}$ = 3.8 Hz, H-1), 4.88 (d, 1H, *J* = 4.6 Hz, OH), 4.58 (d, 1H, *J* = 4.6 Hz, OH), 4.24 (td, 1H, $J_{2,3}$ = 8.3 Hz, $J_{2,F}$ = 51.5 Hz, H-2), 3.89–3.56 (m, 14H, $N(CH_2CH_2Cl)_2$, H-3, H-4, H-5, 2H-6, OH), 2.59 (t, 2H, *J* = 7.5 Hz, $C\mathit{H}_2Ph$), 2.36 (t, 2H, *J* = 7.4 Hz, $COCH_2$), 1.96 (qt, 2H, $CH_2C\mathit{H}_2CH_2$); ^{13}C NMR (50 MHz, acetone-d_6) *δ* 171.45 (NHCO), 153.93 ($C_{Ar}O$), 145.53 ($C_{Ar}N$), 135.49 ($C_{Ar}NH$), 131.43 ($\mathit{C}_{Ar}CH_2$), 130.37, 121.26, 117.68, 113.07 (CH_{Ar}), 99.59 (C-1, $J_{1,F}$ = 23.4 Hz), 93.13 (C-2, $J_{2,F}$ = 184.9 Hz), 77.79 (C-5), 76.11 (C-3, $J_{3,F}$ = 17.1 Hz), 71.21 (C-4, $J_{4,F}$ = 7.9 Hz), 62.31 (C-6), 53.95 (CH_2N), 41.56 (CH_2Cl), 36.93 ($CO\mathit{C}H_2$), 34.86 ($\mathit{C}H_2Ph$), 28.19 ($CH_2\mathit{C}H_2CH_2$); MS (ESI) *m/z* 559.30 $[M+1]^+$; Anal. ($C_{26}H_{33}Cl_2FN_2O_6$) C, H, N.

3.1.19. *N*-{2-[4-(3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosyloxy)phenyl]amino-2-oxoethyl}-4-{4-[bis(2-chloroethyl)amino]phenyl}butanamide (40a). Compound **40a** was prepared according to the procedure described for compound **15a**, starting from amino derivative **39** (1.5 g, 3.28 mmol) and chlorambucil (1.10 g, 3.62 mmol). Purification on silica gel (5:5 to 1:9 cyclohexane/ethyl acetate gradient) gave compound **40a** (2 g, 82%) as a white solid: mp 130 °C; IR(KBr) *ν* 3307, 1755, 1650, 1509, 1367, 1247, 1068, 1044; ^{1}H NMR (200 MHz, $CDCl_3$) *δ* 9.29 (s, 1H, PhN*H*), 7.49 (d, 2H, J_o = 9.0 Hz, H_{Ar}), 7.05–6.96 (m, 4H, H_{Ar}), 6.86 (t, 1H, *J* = 4.6 Hz, $CH_2N\mathit{H}$), 6.59 (d, 2H, J'_o = 8.7 Hz, H_{Ar}), 5.41 (td, 1H, $J_{2,3}$ = $J_{3,4}$ = 9.2 Hz, $J_{3,F}$ = 14.5 Hz, H-3), 5.14–5.05 (m, 2H, H-4, H-1), 4.56 (ddd, 1H, $J_{1,2}$ = 7.8 Hz, $J_{2,F}$ = 50.4 Hz, H-2), 4.29 (dd, 1H, $J_{6a,6b}$ = 12.3 Hz, $J_{5,6a}$ = 5.3 Hz, H-6a), 4.16–4.10 (m, 3H, $C\mathit{H}_2NH$, H-6b), 3.84 (ddd, 1H, $J_{5,6b}$ = 2.3 Hz, $J_{4,5}$ = 9.9 Hz, H-5), 3.73–3.56 (m, 8H, $N(CH_2CH_2Cl)_2$), 2.55 (t, 2H, *J* = 7.4 Hz, $C\mathit{H}_2Ph$), 2.31 (t, 2H, *J* = 7.3 Hz, $COC\mathit{H}_2CH_2$), 2.11, 2.06, 2.05 (each s, 3 × 3H, OAc), 1.93 (qt, 2H, $CH_2C\mathit{H}_2CH_2$); ^{13}C NMR (50 MHz, $CDCl_3$) *δ* 174.18, 170.64, 170.15, 169.65, 167.16 (CO), 153.33 ($C_{Ar}O$), 144.55 ($\mathit{C}_{Ar}N$), 133.75 ($\mathit{C}_{Ar}NH$), 130.36 ($\mathit{C}_{Ar}CH_2$), 129.78, 121.40, 118.12, 112.32 (CH_{Ar}), 99.36 (C-1, $J_{1,F}$ = 23.4 Hz), 89.15 (C-2, $J_{2,F}$ = 190.8 Hz), 72.80 (C-3, $J_{3,F}$ = 19.8 Hz), 72.14 (C-5), 68.18 (C-4, $J_{4,F}$ = 7.3 Hz), 61.93 (C-6), 53.68 (NCH_2), 44.55 ($CO\mathit{C}H_2NH$), 40.64 (CH_2Cl), 35.61 ($CO\mathit{C}H_2CH_2$), 34.10 ($\mathit{C}H_2Ph$), 27.36 ($CH_2\mathit{C}H_2CH_2$), 20.81, 20.69 (CH_3); MS (ESI) *m/z* 742.35 $[M+1]^+$; Anal. ($C_{34}H_{42}Cl_2FN_3O_{10}$) C, H, N.

3.1.20. 4-{4-[Bis(2-chloroethyl)amino]phenyl}butanamide-*N*-{2-[4-(2-deoxy-2-fluoro-β-D-glucopyranosyloxy)phenyl]-amino-2-oxoethyl} (40b). Zemplèn deacetylation as for **17b** and precipitation in EtOH gave compound **40b** (83 mg, 99%) as a white solid: mp 114 °C; IR (KBr) *ν* 3312, 1684, 1510, 1231, 1075; ^{1}H NMR (200 MHz, acetone-d_6)δ 9.22 (br s, 1 H, PhN*H*CO), 7.56 (d, 2 H, J_o = 8.9 Hz, H_{Ar}), 7.43 (br t, 1H, CH_2NHCO), 7.09 (d, 2H, J'_o = 8.6 Hz, H_{Ar}), 7.03 (d, 2H, J_o, H_{Ar}), 6.72 (d, 2H, J'_o, H_{Ar}), 5.19 (dd, 1H, $J_{1,2}$ = 7.9 Hz, $J_{1,F}$ = 2.6 Hz, H-1), 4.88 (br s, 1H, OH), 4.61 (br s, 1H, OH), 4.23 (td, 1H, $J_{2,3}$ = 8.2 Hz, $J_{2,F}$ = 51.2 Hz, H-2), 4.01–3.45 (m, 16H, $N(CH_2CH_2Cl)_2$, H-3, H-4, H-5, 2H-6, CH_2NH, OH), 2.55 (t, 2H, J = 7.6 Hz, CH_2Ph), 2.29 (t, 2H, J = 7.4 Hz, CH_2CH_2CO), 1.89 (qt, 2H, $CH_2CH_2CH_2$); ^{13}C NMR (50 MHz, acetone-d_6) δ 173.83, 168.37 (CO), 154.23 ($C_{Ar}O$), 145.55 ($C_{Ar}N$), 134.78 ($C_{Ar}NH$), 131.56 ($C_{Ar}CH_2$), 130.40, 121.52, 117.73, 113.09 (CH_{Ar}), 99.47 (C-1, $J_{1,F}$ = 23.3 Hz), 93.16 (C-2, $J_{2,F}$ = 184.8 Hz), 77.82 (C-5), 76.09 (C-3, $J_{3,F}$ = 16.8 Hz), 71.18 (C-4, $J_{4,F}$ = 7.6 Hz), 62.27 (C-6), 53.94 (NCH_2), 44.16 ($COCH_2NH$), 41.65 (CH_2Cl), 35.85 ($COCH_2CH_2$), 34.82 (CH_2Ph), 28.40 ($CH_2CH_2CH_2$); MS (ESI) *m/z* 616.31 $[M+1]^+$; Anal. ($C_{28}H_{36}Cl_2FN_3O_7$) C, H, N.

3.2. Biological assays

3.2.1. 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. M4Beu, a human melanoma cell line, was established in the laboratory of Dr. J.F. Doré (INSERM, Unit 218, Lyon, France) from metastatic biopsy specimens and maintained in cell culture for almost 20 years in our lab. Breast cancer adenocarcinoma MCF 7, prostatic adenocarcinoma PC 3, colon adenocarcinoma DLD-1, lung non-small cell carcinoma A 549, ovary adenocarcinoma PA1 human cell lines and L 929 murine cell line were purchased from the European Collection of Cell Cultures (ECACC; Salisbury, United Kingdom).

Stock cell cultures were maintained as monolayers in 75 cm^2 culture flasks in Glutamax Eagle's minimum essential medium with Eagle's salts (MEM; Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal calf serum (Sigma, Saint-Quentin-Fallavier, France), 1 mM sodium pyruvate (Invitrogen), 1X vitamins solution (Invitrogen), 1X non essential amino acids solution (Invitrogen) and 4 μg/ml of gentamicin (Invitrogen). Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

3.2.2. Cell growth inhibition assay. Cells were plated at a density of 5×10^3 cells per well in 96-well microplates (Nunc, Roskilde, Denmark) in 150 μL of culture medium and were allowed to adhere for 16 h before treatment with the compound tested. A 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 4× solution in MEM was then added and a 48 h continuous drug exposure protocol was used. The cytotoxic effect of compounds on tumor cells was then tested using the Resazurin reduction test.

3.2.2.1. Resazurin reduction test. The resazurin reduction test (RRT) was carried out according to the protocol described previously.[63] Briefly, plates were rinsed with 200 μL PBS (37 °C., Gibco) 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 to each well. The plates were incubated for 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 wavelength 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.

3.2.3. Evaluation of cellular stress. L929 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 incubated for 48 h before treatment with the compound tested to reach approximately 90% of confluence. Stock solutions of each compound were prepared in DMSO and kept at −20 °C until use. The percentage of DMSO was kept at 0.5% (v/v) whatever the concentration tested.

3.2.3.1. Evaluation of cellular ROS production. Production of reactive oxygen species (ROS) was evaluated by 2′,7′-dichlofluorescin-diacetate (DCFH-DA; Sigma) assay as previously described with some modifications.[63] Briefly, L929 cells were washed once and incubated in 100 μl of HBSS/Hepes 15 mM, pH 7.4 (HBSS/H) containing 20 μM DCFH-DA for 1 h at 37 °C. After washing, treatment was performed in HBSS/H and the cells were incubated again at 37 °C. Positive control was *tert*-butyl hydroperoxide at 100 μM. Fluorescence was measured after 4 h at 485/530 nm. The results were expressed as the percentage of control. ROS production induction was considered significant when the value was above 150%.

3.2.3.2. Evaluation of cellular reduced glutathione content. GSH content was evaluated as previously described with some modifications.[63] After washing, treatment was performed in HBSS/H and the cells were incubated again at 37 °C for 4 h. Positive control was 100 μM diethylmaleate (DEM, positive control). The cells were then washed with 200 μL of HBSS/H and incubated again for 15 min in 150 μL of HBSS/H containing 7.5 μM monochlorobimane (Invitrogen). Fluo-

rescence was measured at 393/460 nm. The results were expressed as the percentage of control after subtraction of blank values. GSH depletion was considered significant when the value was below 80%.

3.2.3.3. Evaluation of cytolysis. To investigate potential cytolysis induced by the active compounds, we used calcein-AM assay. Briefly, cells were washed once with 200 μL of Hank's balanced salts solution buffered by Hepes 15 mM, pH 7.4 (HBSS/H; Invitrogen). Then, 100 μL of each tested compound at specified concentration or the vehicle (DMSO 0.5% v/v final) or Tween 80 at 0.1 % *m/v* (positive control) in complete medium were added to the well. Tests were done in triplicate. After 4 h of incubation at 37 °C, the absence of detached cells was examined by inverse contrast-phase microscopy and 100 μL of working solution of calcein-AM (4 μM in HBSS/H, Invitrogen) was added. After 1 h of incubation, the fluorescent signal of calcein was read on a fluorometer (Fluoroscan Ascent FL, Labsystem) using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The percentage of membrane-undamaged cells was calculated from the formula: $[(RFU_{sample}–RFU_{100\%})/ (RFU_{vehicle} - RFU_{100\%}] * 100$ where $RFU_{100\%}$ is the value of the background obtained in the 100% cell lysis control (Tween 80 at 0.5% *m/v*). Results are expressed as % of control. Cytolysis was considered significant when this value was below 80%.

Supplementary data

Chemical syntheses, characterization of all intermediates and elemental analysis data of target compounds **14a**, **14c**, **15a–b**, **17a–b**, **20a–b**, **21a–b**, **22a–b**, **31a–b**, **34b**, **37a–b**, and **40a–b**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.03.038.

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