In vitro fluorine-19 nuclear magnetic resonance study of the liberation of antitumor nitrogen mustard from prodrugs

Frédéric Schmidt * and Claude Monneret

UMR 176 CNRS-Institut Curie, Section Recherche 26, rue d'Ulm, 75248 Paris cedex 05, France. E-mail: Frederic.Schmidt@curie.fr; Fax: +33-1/4234-6631; Tel: +33-1/4234-6664

Received (in Cambridge, UK) 19th December 2001, Accepted 25th March 2002
First published as an Advance Article on the web 24th April 2002

The syntheses of two novel glucuronide prodrugs of nitrogen mustard with a fluorine tag are reported. These prodrugs, which differ only by the presence or not of a spacer, were designed for selective activation in the necrotic area of tumors by β-glucuronidase. Kinetics of enzymatic hydrolysis of these prodrugs were determined by ^{19}F-NMR and HPLC *in vitro* studies. The spacer-containing prodrug was found to be the more stable and more quickly cleaved than the other. Thereby it appeared to be a promising candidate for *in vivo* studies.

Introduction

Lack of selectivity is a major limitation in cancer chemotherapy. Indeed, most drugs are not selective enough to be used in optimal dose without severe drawbacks. A solution to this problem would be to target the active drugs to the cancer cells. In this context, a number of protocols using antibody–drug conjugates have been conducted.[1] However, few conjugates have reached the level of clinical trials, primarily due to problems of tumor penetration, internalisation, and antitumor activity of the conjugate.[2]

Alternatively, the ADEPT (Antibody-Directed Enzyme Prodrug Therapy) strategy[3] provides the means to selectively deliver an active drug to its intended target from an inactive prodrug precursor. In this approach, an enzyme is first targetted to the tumor site by monoclonal antibody(Mab)–antigen recognition, and in the second step enzymatic cleavage of the prodrug liberates the active drug at the tumor cell surface. More convenient is the PMT (Prodrug MonoTherapy) strategy[4–6] based on the presence of tumor-associated enzymes[7] such as β-glucuronidase. In normal tissues, this glycosidase is localised in the lysosome compartment of the cell and found in low concentration in the blood. In contrast, it has been shown by enzyme histochemistry that it is specifically liberated extracellularly in necrotic areas. Monocytes and granulocytes are most likely the cells responsible for this effect.[6] Many of the glucuronide prodrugs, such as the anthracycline prodrug HMR 1826, which have been developed for ADEPT have also be used in the PMT approach. From experiments run with human perfused lungs, it appeared that administration of this prodrug led to higher concentration of doxorubicin inside the tumor than those resulting from conventional treatment by doxorubicin itself.[8]

Prodrugs of nitrogen mustards have been developed in the context of the ADEPT strategy.[9,10] Nitrogen mustards are cytotoxic compounds which alkylate DNA.[11,12] Interestingly, these compounds induce less resistance than other classes of anticancer agents, and are highly efficient against quiescent cancer cells. An attractive feature of nitrogen mustard prodrugs is that, whereas the prodrug is stable, the released drug is rapidly transformed into an inactive species (reaction of the nitrogen mustard with a nucleophilic species in the medium). This instability effectively limits the damage to healthy cells in the zone around the tumor.[13]

Different enzymes have been exploited for the cleavage of nitrogen mustard prodrugs. Wallace and Senter used an alkaline phosphatase,[14] and Springer *et al.* focused their studies on carboxypeptidase G2.[15–19] Work by Roffler[20–22] and in our laboratory[23–28] has been centered on the use of β-glucuronidase. Noteworthy is the fact that the only prodrugs based on the ADEPT strategy which have entered clinical trials are prodrugs of nitrogen mustards.[15–17,29]

To develop ADEPT and PMT studies at the preclinical level there is a need to find methods to monitor events *in vivo* in real time. This is particularly relevant for the study of unstable nitrogen mustard drugs. *In vivo* fluorine-19 NMR spectroscopy allows non-invasive real-time identification of administered fluorinated compounds inside human tumors. Furthermore, for kinetic measurements (prodrug : drug ratios) ^{19}F NMR offers advantages, such as a spin of $\frac{1}{2}$, 100% natural abundance, a large chemical-shift dispersion (200 ppm for organofluorine compounds), high detection sensitivity (83% of that of protons), and a low background signal. This technique has been used to study the antitumoral drug 5-FU[27,30–34] but, to date, not for the study of nitrogen mustards.

As the nitrogen mustard prodrugs we have previously synthesised did not contain fluorine,[23] we have prepared two new nitrogen mustards **8** and **18** with a fluorine tag. It must be reminded that some fluorinated nitrogen mustard prodrugs have already been reported by Springer *et al.*,[35] but they widely differ from the prodrugs which are reported in the present study. Indeed, instead of glutamic-containing prodrugs as reported by Springer *et al.*,[35] the prodrugs that constitute the basis of this study are phenol glucuronides. Compound **8** bears an analogy to Roffler's prodrug[20] in that the drug is directly linked to glucuronic acid. In compound **18**, a self-immolative spacer is present between the glucuronic acid and the drug.[23] An attractive design feature in both systems is that the chemical shifts for the free drug and the prodrug form are clearly separated.

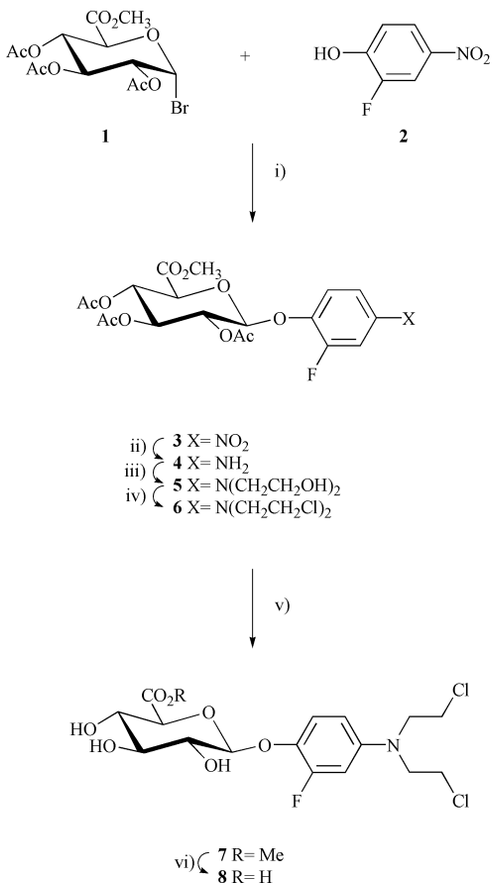
Results and discussion

Synthesis

To obtain prodrug **8**, 2-fluoro-4-nitrophenol **2** was coupled (99%) to the protected bromoglucuronate **1**.[36] The aromatic nitro compound **3** was then converted to the nitrogen mustard **6** in three steps: catalytic hydrogenation to amine **4** (85%), alkylation with ethylene oxide to diol **5** (61%), and transformation of the terminal alcohol functions to the corresponding chlorides *via* mesylation and reaction with LiCl (79%). Finally, hydrolysis

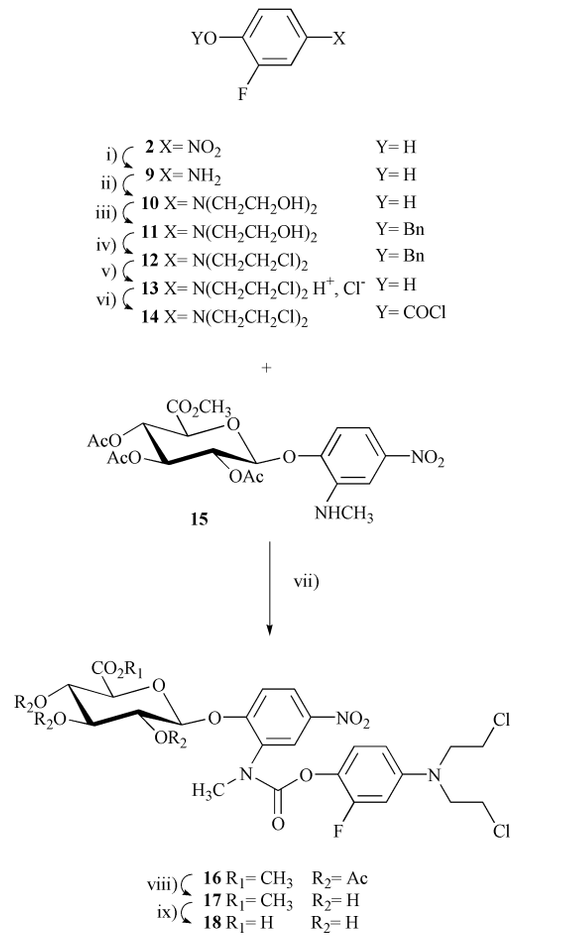
 DOI: 10.1039/b111549a

of the ester groups on the glucuronic acid moiety was effected by treatment with sodium methoxide to give triol **7** (86%), followed by sodium hydroxide to give acid **8** (95%), (Scheme 1).

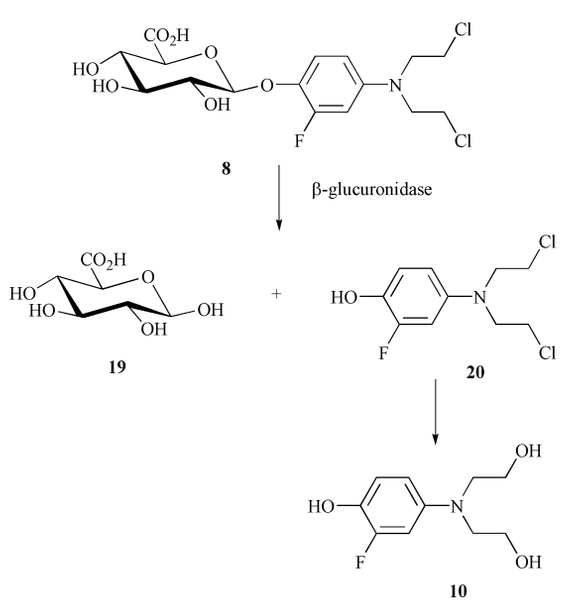


Scheme 1 Synthesis of prodrug **8**. *Reagents and conditions*: i) Ag_2O, acetonitrile (99%); ii) H_2, Pd/C (85%); iii) ethylene oxide, acetic acid (61%); iv) MsCl, LiCl, pyridine (79%); v) MeONa, MeOH (86%); vi) NaOH, H_2O–acetone (95%).

To obtain prodrug **18**, an approach was devised wherein a phenolic nitrogen mustard is coupled to an intermediate in which the glucuronic acid motif is already linked to the aromatic spacer. 2-Fluoro-4-nitrophenol **2** was reduced to 2-fluoro-4-aminophenol[37] **9** by hydrogenation over Pd/C (99%). The amine function in **9** was then alkylated through reaction with ethylene oxide to give **10** (68%), and the phenol function was then protected as the benzyl ether **11** (38%). Conversion of this intermediate to chloride **12** (44%), followed by reductive *O*-debenzylation in acidic media, provided the phenolic nitrogen mustard **13** as its hydrochloride salt. This unstable compound was immediately converted to the corresponding chloroformate **14** by reaction with phosgene in the presence of triethylamine (47%). Chloroformate **14** was then treated with amine **15**[25] in the presence of diisopropylethylamine to give **16** (66%). Two subsequent deprotection steps (77% overall) gave prodrug **18**, *via* **17** (Scheme 2).



Scheme 2 Synthesis of prodrug **18**. *Reagents and conditions*: i) H_2, Pd/C (99%); ii) ethylene oxide, acetic acid (68%); iii) BnBr, KOH, ethanol (38%); iv) MsCl, LiCl, pyridine (44%); v) H_2, HCl, Pd/C (100%); vi) $COCl_2$, NEt_3, THF (47%); vii) $^iPr_2NEt$, THF (66%); viii) MeONa, MeOH (80%); ix) NaOH, H_2O–acetone (96%).



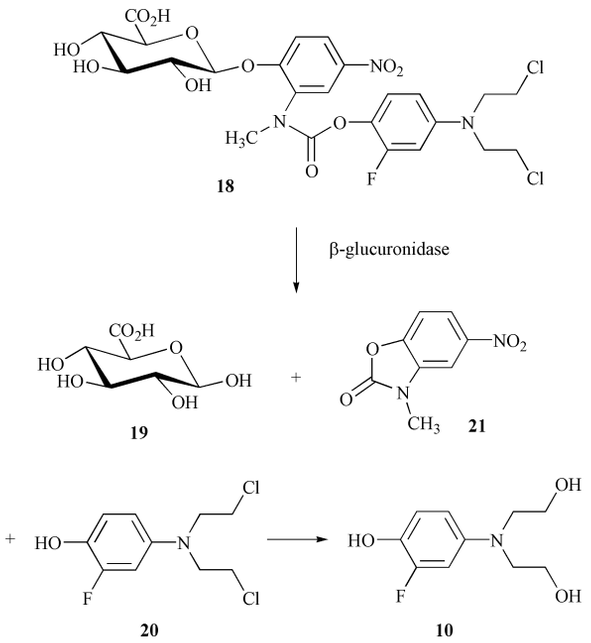
Scheme 3 Cleavage of prodrug **8**.

Liberation of the drug

Both prodrugs **8** and **18** were designed such that the same fluorinated nitrogen mustard mustard **20** (and glucuronic acid **19**) would be liberated in the presence of β-D-glucuronidase (Scheme 3 and 4). For prodrug **18**, the spacer would also undergo cyclisation to give **21** as a secondary product. The released nitrogen mustard **20** was immediately transformed into a more stable compound **10** by reaction with water as the nucleophile.

Cytotoxicity

In comparison with the non-fluorinated nitrogen mustard, the fluorinated analog **20** is slightly less cytotoxic (IC_{50} 15.4 μM *versus* 10.5 μM). The behavior of the fluorinated prodrugs **8** and **18** is similar to that of the corresponding non-fluorinated prodrugs. All these prodrugs are less cytotoxic (IC_{50} > 400 μM) than the corresponding drugs, but, in the presence of



Scheme 4 Cleavage of prodrug **18**.

β-D-glucuronidase, the cytotoxicity of the free drug **20** was restored.

Stability

In the absence of β-D-glucuronidase, we could not detect by HPLC or ^{19}F-NMR any compound resulting from the cleavage of the glycosidic bond. Nevertheless, the two prodrugs **8** and **18** and the free drug **20** were not completely stable in solution. Each nitrogen mustard moiety was hydrolysed to the corresponding diol by displacement of the chlorine atoms.

Their stabilities were measured by HPLC measurements in phosphate buffer at pH 7.2. Interestingly, it was noticed that the transformation of the drug into prodrug led to a stabilisation. Thus, the half-life of the free drug **20** was 14 min whereas those of the prodrugs **8** (without spacer) and **18** (with spacer) were 32, and 170 min, respectively (Fig. 1). The increasing stability can

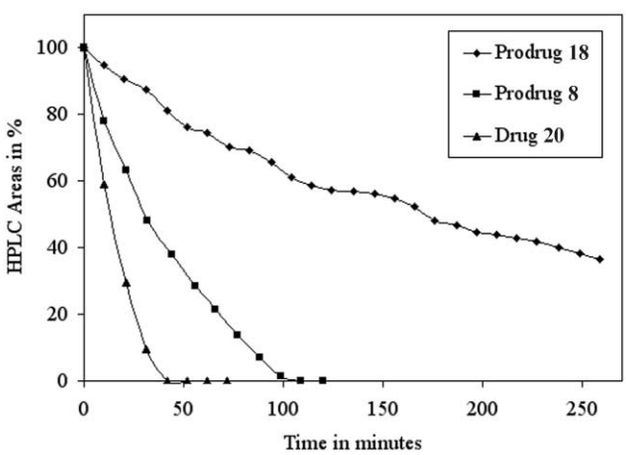


Fig. 1 Stability of prodrugs and free drug.

be related to the decreasing electrophilic character of the nitrogen, promoting the formation of the intermediate aziridinium, as a phenol is more electro-donating than is a glycoside oxygen and much more than is a carbamate oxygen.

^{19}F NMR

Before starting *in vitro* kinetic measurements, different species had to be identified by their ^{19}F-NMR signals in aqueous buffered solution. The chemical shifts were measured with trifluoroacetic acid as the reference[31,38] and all fluorine spectra were recorded with proton decoupling in order to simplify the signals.

The prodrug **8** without spacer gave a signal at δ −55.2 for the fluorine. For the prodrug **18** with spacer, fluorine appears as three signals δ −52.5, −53.0, and −53.2. By heating at 90 °C, the signals coalesced to a unique signal at δ −52.9. The interpretation of that phenomenon is a mixture of rotamers due, in one part, to the bond *cis* or *trans* between the carbamate nitrogen and the carbonyl, and in the other part to another restricted rotation about a different bond. Rotamers could also be detected in ^{1}H-NMR for the N-methyl signal, for instance in the protected prodrug **16**. For the measurements, these three signals were integrated together.

The free nitrogen mustard introduced as its chlorhydrate (hydrochloride) **13** gave a broad signal at δ −59.1. After hydrolysis, the drug gave the dialcohol **10**, which gave a signal at δ −45.7.

All these data indicate that the various signals are different enough to allow their measurement by integration.

Kinetics (NMR and HPLC) of drug release

The kinetics of drug release by β-D-glucuronidase (*E. coli*) from both prodrugs were measured by ^{19}F-NMR and HPLC (UV detection). In all cases, rapid hydrolysis of the glucuronyl moiety occurred with release of the nitrogen mustard **20**.

By fluorine NMR, starting from the prodrug, the signals corresponding to the free nitrogen mustard **20** and the diol **10** resulting from the hydrolysis of the drug appeared and increased (Figs 2 and 3). At the end, only the signal of the

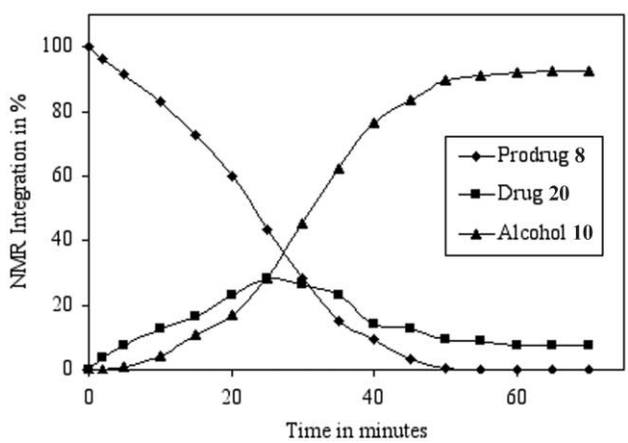


Fig. 2 Hydrolysis of prodrug **8** (NMR measurements).

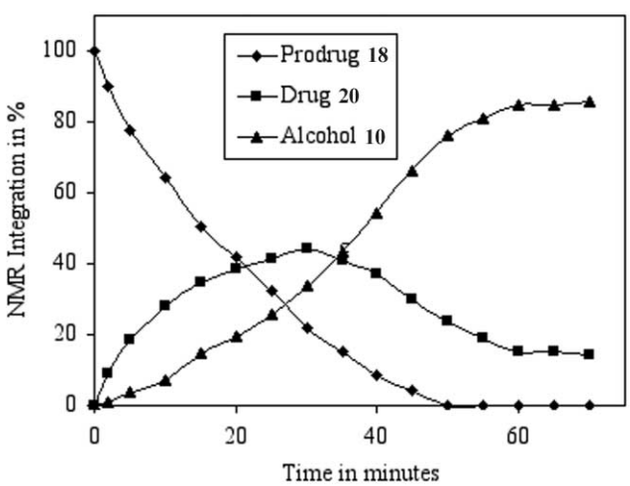


Fig. 3 Hydrolysis of prodrug **18** (NMR measurements).

dialcohol **10** was seen. Comparison between the two prodrugs was also very clear. Without a spacer, the cleavage was slower and needed a higher concentration of enzyme. For the same prodrug concentration, the half-life of **8** with an enzyme concentration of 2000 units mL^{-1} (1527 μg mL^{-1}) was 23 min, and for **18** with an enzyme concentration of 500 units mL^{-1} (382 μg mL^{-1}), the half-life was 15 min.

By HPLC (UV detection), we could clearly compare the concentrations of prodrugs, free drug **20**, and spacer in its cyclised form, **21**, which accumulated. Once again, the prodrug **18** was cleaved faster than was the prodrug **8**. For a concentration of 250 μg mL^{-1}) in prodrug and a concentration of 250 μg mL^{-1}) of β-D-glucuronidase, the half-lives of prodrugs were respectively 7 min for **18**, and 15 min for **8**. The same 15 min half-life for the prodrug **18** could be observed under treatment with a 2.5-less-fold concentration in enzyme.

Conclusions

From these results, it clearly appears that these prodrugs can be used for *in vitro* studies, and consequently for *in vivo* studies. However, among them, the spacer-containing prodrug undoubtly displayed both better stability and easier enzymatic cleavage. For both reasons, such a prodrug was selected for further investigations, in particular by *in vivo* ^{19}F-NMR for the detection and the quantification of the drug in tumors.

Experimental

General

Melting points were taken on a Koffler Bench and are uncorrected. Optical rotations were obtained on a Perkin-Elmer 241 polarimeter. $[\alpha]_D$-Values are given in units of 10^{-1} deg cm^2 g^{-1}. Infrared spectra were measured on a Perkin–Elmer 1710 FT/IR spectrometer. ^{1}H-NMR (300 MHz) spectra were recorded on a Bruker Avance 300 spectrometer (chemical shifts δ in ppm and coupling constants J in Hz). For NMR descriptions, unprimed numbers are used for the glucuronic moiety, primed numbers for the nitrogen mustard, and double-primed numbers for the nitroaromatics. Chemical ionisation mass spectra (CI-MS) were recorded on a NERMAG R10–10C spectrometer. Electrospray ionisation spectra were acquired using a quadrapole instrument with a mass of charge (m/z) range of 2000.

Column chromatography was carried out on Merck silica Kieselgel 60 (230–400 Mesh).

Microanalyses were performed on a Perkin–Elmer 2400 CHN microanalyser.

^{19}F-NMR

^{19}F-NMR (282 MHz) signals were measured on a Bruker Avance 300 at room temperature with an external reference (CF_3CO_2H in D_2O 5% v/v). Proton CPD (Composite Pulse Decoupling) coupling was made possible by a QNI (Quadrupole Nucleus Inverse) Probe (Bruker). The prodrug (concentration 5 mM) was dissolved in D_2O buffered at pH 7.2 by a phosphate buffer (0.02 M).

HPLC Analysis

Analytical HPLC was carried out using a Gilson HPLC system with UV detection at 254 nm. The separation was performed on a reversed-phase column (Zorbax SB-C18, 5 μm, 150 × 4.6 mm) using the isocratic conditions (0.6 mL min^{-1}) of 40% 0.02 M phosphate buffer (pH 3) and 60% acetonitrile.

Stability of compounds in a buffer solution

A solution of 250 μL of prodrug in 0.02 M phosphate buffer (pH 7.2) was incubated for various times at 37 °C. Aliquots were taken at various times and analysed by HPLC after dilution with eluent.

Enzymatic cleavage by *E. coli* β-D-glucuronidase (HPLC)

Hydrolysis was investigated by incubating a solution of 250 μg (mL of prodrug)$^{-1}$ and variable concentrations of *E. coli* β-glucuronidase in 0.02 M phosphate buffer (pH 7.2) at 37 °C. Aliquots (100 μL) were taken at various times and analysed by HPLC after dilution with 300 μL of eluent.

In vitro Cytotoxicity

Cytotoxicity was tested against LoVo cells [human colon cancer cell line obtained from ATCC (Rockville, MD, USA)] using the Methylene Blue assay. The concentration of prodrug or drug inducing 50% of inhibition (IC_{50}) was calculated from the dose–response curve.

Methyl (2-fluoro-4-nitrophenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid)uronate 3

To a solution of methyl 2,3,4-tri-*O*-acetyl-bromo-1-deoxy-α-1-D-glucopyranuronate[36] **1** (16.1 g, 40.5 mmol) in 280 mL of acetonitrile were added silver oxide (22 g, 94.9 mmol) and 2-fluoro-4-nitrophenol **2** (6.27 g, 39.9 mmol). The mixture was stirred overnight at room temperature, filtered over Celite, and concentrated. The product was purified by column chromatography on silica using CH_2Cl_2–MeOH (97.5 : 2.5) as eluent to give the glucuronate **3** (18.8 g, 99%) as a white solid, mp 150 °C (from toluene); $[\alpha]_D^{20}$ −48 (c 0.808 in $CHCl_3$) (Found: C, 48.30; H, 4.26; N, 2.96; F, 4.01. Calc. for $C_{19}H_{20}FNO_{12}$: C, 48.21; H, 4.26; N, 2.93; F, 3.90%); ν_{max}/cm^{-1} ($CDCl_3$) 1761 (CO), 1533, 1351 (NO_2); δ_H (300 MHz; $CDCl_3$; Me_4Si) 8.06–7.99 (2H, m, H3′, H5′), 7.33–7.17 (1H, m, H6′), 5.40–5.26 (4H, m, H1, H2, H3, H4), 4.25 (1H, d, J 8.7, H5), 3.74 (3H, s, CO_2CH_3), 2.10 (3H, s, Ac), 2.07 (3H, s, Ac), 2.06 (3H, s, Ac); m/z (CI, NH_3) 491 $[M + NH_4]^+$.

Methyl (2-fluoro-4-aminophenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid)uronate 4

The nitro derivative (200 mg, 0.423 mmol) **3** was dissolved in 30 mL of ethanol, 10% palladium on carbon (50 mg) was added, and the mixture was hydrogenated overnight at room temperature. After filtration over Celite and evaporation, the product was purified by column chromatography on silica using CH_2Cl_2–MeOH (97.5 : 2.5) as eluent to give the amine **4** (160 mg, 85%) as a white solid, mp 106 °C (from toluene); $[\alpha]_D^{20}$ −39 (c 0.804 in $CHCl_3$); ν_{max}/cm^{-1} ($CDCl_3$) 3694, 3497 (NH), 1758 (CO); δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.02 (1H, dd, $J_{H-F\ (m)}$ 8.9, $J_{H-H\ (o)}$ 8.9, H6′), 6.40 (1H, dd, $J_{H-F\ (o)}$ 12.2, $J_{H-H\ (m)}$ 2.7, H3′), 6.33 (1H, dd, $J_{H-H\ (o)}$ 8.7, $J_{H-H\ (m)}$ 1.8, H5′), 5.32–5.21 (3H, m, H2, H3, H4), 4.85 (1H, d, J 7.3 (β), H1), 4.05 (1H, J 9.0, H5), 3.76 (3H, s, CO_2CH_3), 3.65 (2H, s, NH_2), 2.10 (3H, s, Ac), 2.04 (3H, s, Ac), 2.03 (3H, s, Ac); m/z (CI, NH_3) 461 $[M + NH_4]^+$; (CI, CH_4) 444.130 $[M + H]^+$. $C_{19}H_{23}FNO_{10}$ requires m/z, 444.130.

Methyl {2-fluoro-4-[bis(2-hydroxyethyl)amino]phenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid}uronate 5

To a solution of the amine **4** (1.81 g, 14.1 mmol) in 35 mL of acetic acid at 0 °C was added ethylene oxide (2.42 mL, 48.4 mmol) and the mixture was stirred for 48 hours at room temperature. The acetic acid was evaporated off under vacuum. The solution was neutralised with saturated aqueous sodium bicarbonate. After drying over sodium sulfate and evaporation, the product was purified by column chromatography on silica using EtOAc as eluent to give the dialcohol **5** (1.33 g, 61 %) as a white solid, mp 115 °C (from toluene); $[\alpha]_D^{20}$ −63 (c 0.99 in $CHCl_3$) (Found: C, 51.97; H, 5.64; N, 2.65; F, 3.59. Calc. for

$C_{23}H_{30}FNO_{12}$: C, 51.98; H, 5.69; N, 2.64; F, 3.57%); ν_{max}/cm^{-1} ($CDCl_3$) 3621 (OH), 1758 (CO); δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.09 (1H, dd, $J_{H-F\ (m)}$ 9.1, $J_{H-H\ (o)}$ 9.1, H6′), 6.41 (1H, dd, $J_{H-F\ (o)}$ 14.0, $J_{H-H\ (m)}$ 2.9, H3′), 6.34 (1H, dd, $J_{H-H\ (o)}$ 8.6, $J_{H-H\ (m)}$ 2.8, H5′), 5.35–5.21 (3H, m, H2, H3, H4), 4.85 (1H, d, J 7 (β), H1), 4.03 (1H, J 9.3, H5), 3.84 (4H, t, J 4.8, CH_2OH), 3.76 (3H, s, CO_2CH_3), 3.53 (4H, t, J 4.8, CH_2N), 3.09 (2H, s, OH), 2.09 (3H, s, Ac), 2.04 (3H, s, Ac), 2.02 (3H, s, Ac); *m*/*z* (CI, NH_3) 532 $[M + H]^+$.

Methyl {2-fluoro-4-[bis(2-chloroethyl)amino]phenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid}uronate 6

To a solution of the diol **5** (194 mg, 0.365 mmol) in 2 mL pyridine at 0 °C was added methanesulfonyl chloride (0.15 mL, 0.365 mmol) and the mixture was stirred for 5 min at room temperature, then for 15 min at reflux. Lithium chloride (50 mg, 1.18 mmol) was added and the mixture was kept for 1 hour at reflux. The cooled mixture was poured into aqueous citric acid, extracted with EtOAc, and the extract was dried over sodium sulfate and evaporated. The product was purified by column chromatography on silica using CH_2Cl_2–MeOH (97.5 : 2.5) as eluent to give the dichloride **6** (164 mg, 79%) as a white solid, mp 175 °C (from toluene); $[a]_D^{20}$ −31 (*c* 1.06 in $CHCl_3$) (Found: C, 48.75; H, 5.00; N, 2.46; F, 3.18. Calc. For $C_{23}H_{28}FNO_{10}Cl_2$: C, 48.60; H, 4.97; N, 2.46; F, 3.34%); ν_{max}/cm^{-1} ($CDCl_3$) 1758 (CO); δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.14 (1H, dd, $J_{H-F\ (m)}$ 9.1, $J_{H-H\ (o)}$ 9.1, H6′), 6.41 (1H, dd, $J_{H-F\ (o)}$ 13.6, $J_{H-H\ (m)}$ 3.0, H3′), 6.34 (1H, dd, $J_{H-H\ (o)}$ 8.7, $J_{H-H\ (m)}$ 2.9, H5′), 5.33–5.21 (3H, m, H2, H3, H4), 4.87 (1H, d, J 7.5 (β), H1), 4.05 (1H, J 9.4, H5), 3.77 (3H, s, CO_2CH_3), 3.72–3.66 (4H, m, CH_2Cl), 3.61–3.58 (4H, m, CH_2N), 2.11 (3H, s, Ac), 2.05 (3H, s, Ac), 2.03 (3H, s, Ac); *m*/*z* (CI, NH_3) 568 ($2Cl^{35}$), 570 (Cl^{35}, Cl^{37}), 572 ($2Cl^{37}$) $[M + H]^+$.

Methyl {2-fluoro-4-[bis(2-chloroethyl)amino]phenyl β-D-glucopyranosid}uronate 7

To a solution of **6** (574 mg, 10.1 mmol) in 30 mL of CH_2Cl_2 and 30 mL of MeOH at −15 °C was added sodium methoxide (30 mg, 0.555 mmol) and the mixture was stirred for 4 hours at −15 °C then further sodium methoxide (10 mg, 0.185 mmol) was added and the mixture stirred at −15 °C overnight, neutralised with IRC 50 (H^+) Amberlite, and filtered. The solution was concentrated and the residue was purified by column chromatography on silica using EtOAc as eluent to give **7** (384 mg, 86%) as a white solid, mp 64 °C; $[a]_D^{20}$ −62 (*c* 0.925 in $CHCl_3$) (Found: C, 45.76; H, 5.18; N, 3.12; Calc. for $C_{17}H_{22}FNO_7Cl_2$: C, 46.16; H, 5.01; N, 3.17%); ν_{max}/cm^{-1} (KBr) 3349 (OH), 1746 (CO); δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.12 (1H, dd, $J_{H-F\ (m)}$ 9.1, $J_{H-H\ (o)}$ 9.1, H6′), 6.42 (1H, dd, $J_{H-F\ (o)}$ 12.5, $J_{H-H\ (m)}$ 2.6, H3′), 6.33 (1H, dd, $J_{H-H\ (o)}$ 9.0, $J_{H-H\ (m)}$ 1.5, H5′), 4.72 (1H, d, J 6.1 (β), H1), 4.14 (2H, s, OH), 3.95–3.82 (4H, m, H2, H3, H4, H5), 3.80 (3H, s, CO_2CH_3), 3.74–3.56 (8H, m, CH_2Cl, CH_2N), 1.96 (1H, s,OH); *m*/*z* (CI, NH_3) 442 ($2Cl^{35}$), 444 (Cl^{35}, Cl^{37}), 446 ($2Cl^{37}$) $[M + H]^+$.

2-Fluoro-4-[bis(2-chloroethyl)amino]phenyl β-D-glucopyranosiduronic acid 8

To a solution of ester **7** (70 mg, 0.158 mmol) in 5 mL of acetone at −15 °C was added 1 M sodium hydroxide (0.5 mL, 0.5 mmol), the solution was stirred for 5 min at −15 °C, neutralised with 1 M HCl, and evaporated. The residue was purified by column chromatography on silica using CH_3CN–H_2O (80 : 20) as eluent to give, after lyophilisation, acid **8** (64 mg, 95%) as a white solid, mp 220 °C (dec.); $[a]_D^{20}$ −48 (*c* 0.456 in CH_3OH); ν_{max}/cm^{-1} (KBr) 3355 (OH), 1738 (CO); δ_H (300 MHz; CD_3OD; Me_4Si) 7.19 (1H, dd, $J_{H-F\ (m)}$ 9.4, $J_{H-H\ (o)}$ 9.4, H6′), 6.53 (1H, dd, $J_{H-F\ (o)}$ 14.2, $J_{H-H\ (m)}$ 2.9, H3′), 6.43 (1H, dd, $J_{H-H\ (o)}$ 8.8, $J_{H-H\ (m)}$ 2.9, H5′), 3.69–3.49 (13H, m, H1, H2, H3, H4, H5, CH_2Cl, CH_2N); δ_F (382 MHz; D_2O buffered; TFA) −55.2; *m*/*z* (ES) 450 ($2Cl^{35}$), 452 (Cl^{35}, Cl^{37}), 454 ($2Cl^{37}$) $[M + Na]^+$; (ES) 427.061 ($2Cl^{35}$). $C_{16}H_{20}FNO_7Cl_2$ requires *M*, 427.060.

2-Fluoro-4-[bis(2-hydroxyethyl)amino]phenol 10

To a solution of the aminophenol **9**[37] (4.0 g, 31.5 mmol) in 85 mL of acetic acid and 85 mL of water at 0 °C was added ethylene oxide (15 mL, 300 mmol) and the mixture was stirred at room temperature for 72 hours. The acetic acid was evaporated off under vacuum. The product was purified by two successive column chromatographic separations on silica using EtOAc for the first and CH_2Cl_2–MeOH (9 : 1) for the second eluent, giving the triol **10** (4.83 g, 68%) as a pink gum which turned brown within a few minutes. We did not obtain a sample suitable for microanalysis and used this crude compound in the next step. ν_{max}/cm^{-1} (KBr) 3392 (OH); δ_H (300 MHz; DMSO; Me_4Si) 6.78 (1H, dd, $J_{H-F\ (m)}$ 9.2, $J_{H-H\ (o)}$ 9.2, H6′), 6.70–6.10 (2H, H3′, H5′), 4.72 (2H, OH), 3.67–3.15 (8H, OCH_2, NCH_2); δ_F (382 MHz; D_2O buffered; TFA) −45.7; *m*/*z* (CI, CH_4) 216.104 $[M + H]^+$. $C_{10}H_{15}FNO_3$ requires *m*/*z*, 216.104.

1-Benzyloxy-2-fluoro-4-[bis(2-hydroxyethyl)amino]benzene 11

To a solution of the triol **10** (3.71 g, 17.2 mmol) in 35 mL of 95% ethanol was added benzyl bromide (2.06 mL, 17.3 mmol), then commercial 85% potassium hydroxide pellets (1.14 g, 17.3 mmol) were added and the mixture was stirred for 2 hours at reflux, evaporated, the residue was taken up in EtOAc, and the solution was washed with water. The organic phase was dried over sodium sulfate, evaporated, and purified by column chromatography on silica using EtOAc as eluent to give the benzyl ether **11** (3.71 g, 38%) as a viscous brown liquid; ν_{max}/cm^{-1} ($CDCl_3$) 3404 (OH); δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.50–7.20 (5H, br, Ph), 6.89 (1H, dd, $J_{H-F\ (m)}$ 9.2, $J_{H-H\ (o)}$ 9.2, H6′), 6.50 (1H, dd, $J_{H-F\ (o)}$ 14.3, $J_{H-H\ (m)}$ 3.0, H3′), 6.35 (1H, ddd, $J_{H-H\ (o)}$ 9.2, $J_{H-H\ (m)}$ 3.0, $J_{H-F\ (p)}$ 1.1, H5′), 5.04 (2H, s, CH_2Ph), 3.81 (4H, t, J 4.8, OCH_2), 3.49 (4H, t, J 4.7, NCH_2), 3.11 (2H, s, OH); *m*/*z* (CI, NH_3) 306 $[M + H]^+$.

1-Benzyloxy-2-fluoro-4-[bis(2-chloroethyl)amino]benzene 12

To a solution of the diol **11** (1.68 g, 5.5 mmol) in 11 mL of pyridine at 0 °C was added methanesulfonyl chloride (1.66 mL, 21.4 mmol) and the mixture was stirred for 5 min at room temperature, then for 15 min at reflux. Lithium chloride (0.33 g, 7.79 mmol) was added and the mixture was kept 15 min at reflux. The cooled mixture was poured into aqueous citric acid, extracted with EtOAc, and the extract was dried over sodium sulfate and evaporated. The product was purified by column chromatography on silica using cyclohexane–EtOAc (3 : 1) as eluent and crystallisation from petroleum ether (80–110 °C) to give the dichloride **12** (824 mg, 44%) as a beige solid, mp 76 °C (Found: C, 59.84; H, 5.44; N, 3.94; F, 5.32. Calc. for $C_{17}H_{18}FNOCl_2$: C, 59.66; H, 5.30; N, 4.09; F, 5.55%); ν_{max}/cm^{-1} ($CDCl_3$) 1519; δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.46–7.23 (5H, br, Ph), 6.91 (1H, dd, $J_{H-F\ (m)}$ 9.2, $J_{H-H\ (o)}$ 9.2, H6′), 6.49 (1H, dd, $J_{H-F\ (o)}$ 14.0, $J_{H-H\ (m)}$ 3.0, H3′), 6.33 (1H, ddd, $J_{H-H\ (o)}$ 8.9, $J_{H-H\ (m)}$ 2.9, $J_{H-F\ (p)}$ 1.2, H5′), 5.06 (2H, s, CH_2Ph), 3.70–3.58 (8H, m, NCH_2, CH_2Cl); *m*/*z* (CI, NH_3) 342 ($2Cl^{35}$), 344 (Cl^{35}, Cl^{37}), 346 ($2Cl^{37}$) $[M + H]^+$.

2-Fluoro-4-[bis(2-chloroethyl)ammonio]phenol chloride 13†

Dry HCl gas was bubbled into a suspension of the benzyl ether **12** (710 mg, 2.08 mmol) in absolute ethanol until dissolution. Palladium on carbon (10%; 0.2 g) was added and the compound was hydrogenated for 3 hours. The mixture was filtered and evaporated under vacuum at room temperature to give the

† Strictly speaking, the center takes precedence for principal group:**13** is bis(2-chloroethyl)(3-fluoro-4-hydroxyphenyl)ammonium chloride

salt (600 mg, quantitative) as a white solid. Compound **13** was utilised as soon as possible without any further purification as it is rather unstable, especially in solution. ν_{max}/cm^{-1} ($CDCl_3$) 3404 (OH), 1525; δ_H (300 MHz; DMSO; Me_4Si) 6.85 (1H, dd, $J_{H-F\ (m)}$ 9.3, $J_{H-H\ (o)}$ 9.3, H6′), 6.63 (1H, dd, $J_{H-F\ (o)}$ 14.4, $J_{H-H\ (m)}$ 2.7, H3′), 6.44 (1H, dd, $J_{H-H\ (o)}$ 8.8, $J_{H-H\ (m)}$ 2.0, H5′), 3.71–3.60 (8H, m, NCH_2, CH_2Cl); δ_F (382 MHz; D_2O buffered; TFA) −59.1; *m/z* (CI, NH_3) 252 ($2Cl^{35}$), 254 (Cl^{35}, Cl^{37}), 256 ($2Cl^{37}$) $[MH]^+$.

Methyl {2-[({2-fluoro-4-[bis(2-chloroethyl)amino]phenoxy}-carbonyl)methylamino]-4-nitrophenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid}uronate 16

To a suspension of chloride **13** (556 mg, 1.93 mmol) in anhydrous THF at 0 °C was added a 20% solution of phosgene in toluene (8.2 mL), followed by triethylamine (1 mL, 7.17 mmol). The mixture was stirred for 1 hour at 0 °C, then for 2 hours at room temperature. After filtration under argon and evaporation at room temperature under vacuum, the compound was purified by column flash chromatography on silica using CH_2Cl_2 as eluent to give the chloroformate **14** (282 mg, 47%) as a pale pink liquid. This reactive compound was utilised immediately as it cannot be kept in the absence of excess of phosgene. Any attempt to characterise this product resulted in loss of the chloroformate function.

To a solution of freshly prepared chloroformate **14** (282 mg, 0.896 mmol) in 10 mL of THF were added the aniline derivative **15**[25] (400 mg, 0.826 mmol) and diisopropylethylamine (0.25 mL, 1.44 mmol). The solution was brought to reflux for 2 hours and was then evaporated. The product was purified by column chromatography on silica using CH_2Cl_2–MeOH (95 : 5) to give **16** (415 mg, 66%) as a white solid, mp 108 °C; $[\alpha]_D^{20}$ −41 (*c* 0.888 in $CHCl_3$) (Found: C, 48.83; H, 4.59; N, 5.45; Calc. for $C_{31}H_{34}FN_3O_{14}Cl_2$: C, 48.83; H, 4.49; N, 5.51%); ν_{max}/cm^{-1} ($CDCl_3$) 1759 (CO), 1520, 1351 (NO_2); δ_H (300 MHz; $CDCl_3$; Me_4Si) 8.28–8.19 (2H, m, H3″, H5″), 7.41 (1H, d, *J* 8.7, H6″), 7.00 (1H, dd, $J_{H-F\ (m)}$ 8.9, $J_{H-H\ (o)}$ 8.9, H6′), 6.42–6.34 (2H, m, H3′, H5′), 5.45–5.30 (4H, m, H1, H2, H3, H4), 4.29 (1H, d, *J* 9.2, H5), 3.77–3.59 (11H, m, OCH_3, NCH_2, CH_2Cl), 3.40, 3.27 (3H, 2s, NCH_3 rotamers) 2.14–2.02 (9H, m, OAc); *m/z* (CI, NH_3) 762 ($2Cl^{35}$), 764 (Cl^{35}, Cl^{37}), 766 ($2Cl^{37}$) $[M + H]^+$.

Methyl{2-[({2-fluoro-4-[bis(2-chloroethyl)amino]phenoxy}-carbonyl)methylamino]-4-nitrophenyl β-D-glucopyranosid}-uronate 17

To a solution of **16** (212 mg, 0.278 mmol) in 10 mL of MeOH at −20 °C was added sodium methoxide (5 mg, 0.0925 mmol) and the mixture was stirred for 6 hours at −20 °C, neutralised with IRC 50 (H^+) Amberlite, and filtered. The solution was concentrated and the residue was purified by column chromatography on silica using EtOAc as eluent to give **17** (361 mg, 80%) as a white solid, mp 96 °C; $[\alpha]_D^{20}$ −68 (*c* 1.01 in $CHCl_3$); ν_{max}/cm^{-1} ($CDCl_3$) 3438 (OH), 1724 (CO), 1521, 1350 (NO_2); δ_H (300 MHz; $CDCl_3$; Me_4Si) 8.23–8.14 (2H, m, H3″, H5″), 7.22 (1H, d, *J* 8.8, H6″), 7.10–6.92 (1H, m, H6′), 6.53–6.38 (2H, m, H3′, H5′), 5.07 (1H, br, H1), 4.21 (1H, s, OH), 4.14–4.04 (1H, m, H5), 3.89–3.25 (17H, m, OCH_3, NCH_3, NCH_2, CH_2Cl, H2, H3, H4), 1.84 (2H, s, OH); *m/z* (CI, NH_3) 636 ($2Cl^{35}$), 638 (Cl^{35}, Cl^{37}), 640 ($2Cl^{37}$) $[M + H]^+$.

2-[({2-Fluoro-4-[bis(2-chloroethyl)amino]phenoxy}carbonyl)-methylamino]-4-nitrophenyl β-D-glucopyranosiduronic acid 18

To a solution of ester **17** (87 mg, 0.137 mmol) in 10 mL of acetone at −15 °C was added 1 M sodium hydroxide (0.3 mL, 0.3 mmol), and the solution was stirred for 15 min at −15 °C, neutralised with 1 M HCl, and evaporated. The residue was purified by column chromatography on silica using CH_3CN–H_2O (80 : 20) as eluent to give, after lyophilisation, **18** (82 mg, 96%) as a white solid, mp 180 °C; $[\alpha]_D^{20}$ −43 (*c* 0.174 in MeOH); ν_{max}/cm^{-1} (KBr) 3418 (OH), 1718 (CO), 1520, 1350 (NO_2); δ_H (300 MHz; CD_3OD; Me_4Si)) 8.28–8.22 (2H, m, H3″, H5″), 7.52 (1H, d, *J* 8.8, H6″), 7.20–7.05 (1H, m, H6′), 6.56–6.43 (2H, m, H3′, H5′), 5.21 (1H, br, H1), 3.87 (1H, d, *J* 9.1, H5), 3.72–3.26 (14H, m, NCH_3, NCH_2, CH_2Cl, H2, H3, H4); δ_F (382 MHz; D_2O buffered; TFA) −52.5, −53.0, −53.2 (rotamers); *m/z* (ES) 644 ($2Cl^{35}$), 646 (Cl^{35}, Cl^{37}), 648 ($2Cl^{37}$) $[M + Na]^+$; (ES) 621.093 ($2Cl^{35}$). $C_{24}H_{26}FN_3O_{11}Cl_2$ requires *M*, 621.093.

Acknowledgements

We would like to thank Hoechst Marion Roussel for cytotoxicity measurements and Mrs Ioana Ungureanu for the synthesis of some compounds during her one-month training in our laboratory.

References

1 D. Blakey, *Acta Oncol.*, 1992, **31**, 91–97.
2 R. Jain, *Cancer Res.*, 1987, **47**, 3039–3051.
3 K. Bagshawe, *Br. J. Cancer*, 1987, **56**, 531–532.
4 K. Bosslet, R. Straub, M. Gerken, H. Petrul, J. Czech, J.-C. Florent, M. Koch, F. Tillequin, F. Schmidt, C. Monneret and J.-P. Gesson, in *Neue Therapeutische Prinzipien in der Krebsbehandlung. 4. Salzburger Symposium zur Lebensqualitaet Chronisch Kranker*, Georg Thieme Verlag, Stuttgart and New York, 1996, pp. 123–130.
5 K. Bosslet, J. Czech and D. Hoffmann, *Tumor Targeting*, 1995, **1**, 45–50.
6 K. Bosslet, R. Straub, M. Blumrich, J. Czech, M. Gerken, B. Sperker, H. K. Kroemer, J.-P. Gesson, M. Koch and C. Monneret, *Cancer Res.*, 1998, **58**, 1195–1201.
7 A. Sinhababu and D. Thakker, *Adv. Drug Deliv. Rev.*, 1996, **19**, 241–273.
8 T. Murdter, B. Sperker, K. Kivistö, M. McClellan, P. Fritz, G. Friedel, A. Linder, K. Bosslet, H. Toomes, R. Diekesmann and H. Kroemer, *Cancer Res.*, 1997, **57**, 2440–2445.
9 C. Springer and I. Niculescu-Duvaz, *Anti-Cancer Drug Des.*, 1995, **10**, 361–372.
10 P. Burke, *Adv. Drug Deliv. Rev.*, 1996, **22**, 331–340.
11 S. Rink, M. Salomon, M. Taylor, S. Rajur, L. McLaughlin and P. Hopkins, *J. Am. Chem. Soc.*, 1993, **115**, 2551–2557.
12 G. Bauer and L. Povirk, *Nucleic Acids Res.*, 1997, **25**, 1211–1218.
13 D. Blakey, D. Davies, R. Dowell, S. East, P. Burke, S. Sharma, C. Springer, A. Mauger and R. Melton, *Br. J. Cancer*, 1995, **72**, 1083–1088.
14 P. Wallace and P. Senter, *Bioconjugate Chem.*, 1991, **2**, 349–352.
15 C. Springer, G. Poon, S. Sharme and K. Bagshawe, *Cell Biophys.*, 1993, **22**, 9–26.
16 C. Springer, R. Dowell, P. Burke, E. Hadley, D. Davies, D. Blakey, R. Melton and I. Niculescu-Duvaz, *J. Med. Chem.*, 1995, **38**, 5051–5065.
17 J. Martin, S. Stribbling, G. Poon, R. Begent, M. Napier, S. Sharma and C. Springer, *Cancer Chemother. Pharmacol.*, 1997, **40**, 189–201.
18 S. Stribbling, J. Martin, R. Pedley, J. Boden, S. Sharma and C. Springer, *Cancer Chemother. Pharmacol.*, 1997, **40**, 277–284.
19 D. Niculescu-Duvaz, I. Niculescu-Duvaz, F. Friedlos, J. Martin, R. Spooner, L. Davies, R. Marais and C. Springer, *J. Med. Chem.*, 1998, **41**, 5297–5309.
20 S. Wang, J. Chern, M. Yeh, J. Ng, E. Tung and S. Roffler, *Cancer Res.*, 1992, **52**, 4484–4491.
21 T. Chen, S. Wei, B. Chen, J. Chern, M. Wu, P. Liu and S. Roffler, *Br. J. Cancer*, 1999, **79**, 1378–1385.
22 T. Cheng, W. Chou, B. Chen, J. Chern and S. Roffler, *Biochem. Pharmacol.*, 1999, **58**, 325–328.
23 F. Schmidt, J.-C. Florent, C. Monneret, R. Straub, J. Czech, M. Gerken and K. Bosslet, *Bioorg. Med. Chem. Lett.*, 1997, **7**, 1071–1076.
24 F. Schmidt, J.-C. Florent and C. Monneret, *C. R. Acad. Sci., Ser. IIc: Chim.*, 1998, **1**, 421–424.
25 S. Desbene, H. van Dufat-Trinh, S. Michel, F. Tillequin, M. Koch, F. Schmidt, J.-C. Florent, C. Monneret, R. Straub, J. Czech, M. Gerken and K. Bosslet, *Anti-Cancer Drug Des.*, 1999, **14**, 93–106.
26 J.-C. Florent, X. Dong, G. Gaudel, S. Mitaku, C. Monneret, J.-P. Gesson, J.-C. Jacquesy, M. Mondon, B. Renoux, S. Andrianomenjanahary, S. Michel, M. Koch, F. Tillequin, M. Gerken, J. Czech, R. Straub and K. Bosslet, *J. Med. Chem.*, 1998, **41**, 3572–3581.

27 R. Madec-Lougerstay, J.-C. Florent and C. Monneret, *J. Chem. Soc., Perkin Trans. 1*, 1999, 1369–1375.
28 F. Schmidt, I. Ungureanu, R. Duval, A. Pompon and C. Monneret, *Eur. J. Org. Chem.*, 2001, 2129–2134.
29 K. Bagshawe, S. Sharma, P. Burke, R. Melton and R. Knox, *Curr. Opin. Immunol.*, 1999, **11**, 579–583.
30 J.-L. Guerquin-Kern, A. Volk, E. Chenu, R. Lougerstay-Madec, C. Monneret, J.-C. Florent, D. Carrez and A. Croisy, *NMR Biomed.*, 2000, **13**, 306–310.
31 J.-L. Guerquin-Kern, F. Leteurtre, A. Croisy and J.-M. Lhoste, *Cancer Res.*, 1991, **51**, 5770–5773.
32 P. Price and J. Griffiths, *Lancet*, 1994, **343, ii**, 1174–1175.
33 C. Presant, W. Wolf, V. Waluch, C. Wiseman, P. Kennedy, D. Blayney and R. Brechner, *Lancet*, 1994, **343, ii**, 1184–1187.
34 E. Aboagye, D. Artemov, P. Senter and Z. Bhujwalla, *Cancer Res.*, 1998, **58**, 4075–4078.
35 C. Springer, I. Niculescu-Duvaz and R. Pedley, *J. Med. Chem.*, 1994, **37**, 2361–2370.
36 G. Bollenback, J. Long, D. Benjamin and J. Lindquist, *J. Am. Chem. Soc.*, 1955, **77**, 3310–3315.
37 D. Aymes and M. Paris, *Bull. Soc. Chim. Fr.*, 1980, **2**, 175–178.
38 P. Hansen, H. Dettman and B. Sykes, *J. Magn. Reson.*, 1985, **62**, 487–496.