Two novel nucleoside ester derivatives of chlorambucil as potential antileukemic prodrugs: a preliminary study

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2-Chloro-2′-deoxyadenosine (cladribine) and chlorambucil are two drugs used in the treatment of lymphoid malignancies. We have synthesized 5′-*O*-esters of cladribine and its parental nucleoside 2′-deoxyadenosine with chlorambucil (2-chloro-2′-deoxyadenosine–chlorambucil and 2′-deoxyadenosine–chlorambucil, respectively) and compared some properties of the esters with regard to their potential use as antileukemic prodrugs. The 5′-*O*-ester bond showed no spontaneous hydrolysis at pH 7.4, but was susceptible to hydrolysis by porcine liver esterase and enzymes present in human lymphocyte lysate and blood plasma. Both 2-chloro-2′-deoxyadenosine–chlorambucil and 2′-deoxyadenosine–chlorambucil were taken up more avidly than their parental nucleosides by normal and malignant human lymphoid cells. 2-Chloro-2′-deoxyadenosine–chlorambucil was by an order of magnitude more toxic than 2′-deoxyadenosine–chlorambucil to human leukemic MOLT4 cells in culture. On the other hand, 2-chloro-2′-deoxyadenosine–chlorambucil cytotoxicity did not exceed that of its parental 2-chloro-2′-deoxyadenosine in MOLT4 cells, whereas 2′-deoxyadenosine–chlorambucil was considerably more cytotoxic than free chlorambucil in a variety of myeloid and lymphoid human malignant cell lines. Moreover, acute toxicity of 2′-deoxyadenosine–chlorambucil was lower than that of chlorambucil in mice. In summary, 2′-deoxyadenosine–chlorambucil, but not 2-chloro-2′-deoxyadenosine–chlorambucil, shows promise for clinical utility as a chlorambucil prodrug and thus warrants a more detailed study *in vivo*. *Anti-Cancer Drugs* 18:301–310 © 2007 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2007, **18**:301–310

Keywords: chlorambucil, cladribine, cytotoxicity, 2′-deoxyadenosine, nucleoside esters, oncohematological diseases, prodrug

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Sponsorship: The study was supported by the Foundation for the Development of Diagnostics and Therapy, Warsaw, Poland.

Received 23 August 2006 **Revised form accepted** 27 September 2006

Introduction

The major weakness of cancer chemotherapy is that most anticancer drugs do not distinguish neoplastic from normal proliferating cells and their toxicity to the latter limits dosage; this may render the therapy ineffective or less effective. The need to improve selectivity and reduce unwanted side-effects of pharmacotherapy have led to new therapeutic strategies, one of which is the use of drug precursors, or prodrugs, that may be inactive *per se*, but preferentially accumulate and/or are converted to active agents in cancer cells. Such constructs may include a carrier moiety that, in addition to properly targeting the agent [1], may have or generate a useful activity of its own.

Chlorambucil {4-[4-bis(2-chloroethyl)-aminophenyl] butyric acid, CLB} and 2-chloro-2′-deoxyadenosine (cladribine, 2CdA) are the two drugs used mostly in lymphoid malignancies. CLB is a long-known alkylating agent used as the first-line therapy in B-cell chronic lymphocytic leukemia (CLL) and occasionally also in solid neoplasms [2]. 2CdA, an analog of the endogenous purine nucleoside 2′-deoxyadenosine (dA), is selectively toxic to normal and transformed lymphocytes owing to some enzymatic peculiarities of the cells [3]. It is used as a first-line therapy in hairy cell leukemia and for the primary or rescue treatment of patients with some chronic lymphoid neoplasms, mostly B-cell CLL, low-grade non-Hodgkin B-cell and T-cell lymphomas, and Waldenström's macroglobulinemia [4–7]. The mechanisms of action of purine nucleoside analogs and alkylating agents differ, creating a possibility for additive effects or synergism. The reports on in-vitro effects of combined treatment with 2CdA and CLB were, however, inconsistent [8–10]. Moreover, some promising results were initially reported in oncohematologic patients treated

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with a combination of cladribine and an alkylating cytostatic [11–17], but recent reports showed no long-term advantage of the combinations over 2CdA monotherapy [18,19].

Here, we report on the synthesis and preliminary characteristics of CLB esters with 2CdA and dA with regard to their potential use as anticancer agents. The aim of the study was to make a dual prodrug that would release both its parental cytostatics at their target site. As the CLB molecule carries a carboxyl group, joining it with 2CdA via a 5′-*O*-ester bond seemed enticing both for apparent feasibility of the synthesis and the expected unproblematic reconversion of the ester to its parental drugs by esterases present in malignant cells [20,21]. This latter process should uphold the transmembrane gradient of ester concentration and allow continuing undisturbed influx of the ester, and hence facilitate delivery of the ester and its conversion into active agents at their target. The presence of the purine nucleoside moiety in the ester molecule may also enable the influx of the ester via nucleoside transporters present in the cell membrane. This would be especially advantageous for intracellular CLB delivery, as free CLB enters the cells by less-efficient passive diffusion [22] and might increase the toxicity of the ester above that of a simple equimolar combination of the ester parental drugs. The 5′-*O*-ester of CLB and dA was included in this study for comparison, as a model compound carrying a natural purine nucleoside moiety with little toxicity of its own.

Materials and methods

Chemicals

CLB (98% purity) was purchased from Sigma-Aldrich (St Louis, Missouri, USA), and 2CdA was donated by the Foundation for the Development of Diagnostics and Therapy (Warsaw, Poland). [8-^{14}C]2CdA (55 Ci/mol specific activity, 99.8% radiochemical purity, 0.1 mCi/ml) and [8-^{14}C]dA (56 Ci/mol specific activity, 99.5% purity, 0.1 mCi/ml) were purchased from Moravek Biochemicals (Brea, California, USA). Analytical grade acetonitrile, dimethyl sulfoxide (DMSO) and silica gel 60 (200–400 mesh) were purchased from Merck (Darmstadt, Germany), NCS-II Tissue Solubilizer from Amersham Biosciences (Little Chalfont, UK), Lymphoprep from AxisShield (Oslo, Norway), and tissue culture media from Gibco (Grand Island, New York, USA) and the Institute of Immunology and Experimental Therapy (Wrocław, Poland). Precoated 0.25-mm silica gel F_{254} plates for analytical thin-layer chromatography (TLC) were obtained from Merck. All other chemicals were purchased from Sigma-Aldrich and were of analytical grade.

Synthesis of 5′-*O*-esters

General procedure

A mixture of dA or 2CdA and CLB (1.5 mmol each) was evaporated twice with pyridine (10 ml) and suspended in dry pyridine (12 ml). To the stirred suspension, triphenylphosphine (525 mg, 2 mmol) and diisopropyl azodicarboxylate (405 mg, 2 mmol) were added portion-wise over 30 min at room temperature. The final mixture was stirred for an additional 30 min and evaporated to oil that was coevaporated three times with toluene to remove the remainder of pyridine. The residue was applied onto a silica gel 60 column (3 × 25 cm) and chromatographed with CH_2Cl_2 (400 ml) followed by a CH_2Cl_2–MeOH mixture (95 : 5, v/v). The product-containing fractions were evaporated to solid foam.

5′-O-chlorambucilyl-2-chloro-2′-deoxyadenosine (2-chloro-2′-deoxyadenosine–chlorambucil)

From 2CdA and CLB. Yield: 49%. TLC: R_f (MeOH/$CHCl_3$, 1 : 9, v/v): 0.49. ^{1}H-nuclear magnetic resonance (NMR) ($CDCl_3$): δ 8.31 (1H, s); 6.99 and 6.64 (4H, 2d), 6.36 (1H, dd, J 6.1 and 6.3); 4.46 (1H, m); 4.16 and 4.32 (2H, m); 4.00 (1H, m); 4.22 (1H, m); 3.68 (8H, m); 1.73-2.72 (2H, 2 m). Electrospray ionization-time-of-flight mass for $C_{25}H_{31}Cl_3N_6O_4$ ($M + H^+$): calculated: 571.1389; found: 571.1376. Analysis for $C_{24}H_{29}Cl_3N_6O_4$ (571.90): calculated: C, 50.41; H, 5.11; N, 14.69; found: C, 50.29; H, 5.15; N, 14.52.

5′-O-chlorambucilyl-2′–deoxyadenosine (2′-deoxyadenosine–chlorambucil)

From dA and CLB. Yield: 56%. TLC: R_f (MeOH/$CHCl_3$, 1 : 9, v/v): 0.44. ^{1}H-NMR ($CDCl_3$): δ 8.30 (1H,s), 8.15 (1H, s); 6.64 and 6.98 (4H, 2d); 6.36 (1H, dd, J 6.1 and 6.3); 4.47 (1H, m); 4.18, 4.25 (2H, m); 4.00 (1H, m); 3.68 (8H, m); 1.74, 2.80 (2H, m). Electrospray ionization-time-of-flight mass for $C_{25}H_{32}Cl_2N_6O_4$ ($M + Na^+$): calculated: 559.1598; found 559.1582. Analysis for $C_{25}H_{32}Cl_2N_6O_4$ (537.45): calculated: C, 53.64; H, 5.63; N, 15.64; found: 53.57; H, 5.55; N, 15.54.

Mass spectra were measured on an Applied Biosystems Mariner ESI-TOF instrument (Foster City, California, USA). ^{1}H-NMR spectra (in p.p.m.) were acquired at 298 K in $CDCl_3$ using a Jeol LA 400 spectrometer (Peabody, Massachusetts, USA) and tetramethylsilane as internal standard.

Human peripheral blood plasma and mononuclear cells

Peripheral blood was taken from healthy volunteers, with informed consent of the donors, using heparin as an anticoagulant. One half of each sample was spun at 3000 *g* for 10 min to give plasma. The other half was used to obtain peripheral blood mononuclear cells (PBMCs), employing Lymphoprep according to the manufacturer's instructions. The PBMC suspension obtained was washed three times by adding nine volumes of physiological saline, spinning for 10 min at 600 *g*, 4°C, and discarding the supernatant. The final pellet was kept at −70°C for later use or was instantly lysed by three cycles of freezing and thawing (−70/+37°C). The lysate was

cleared of cell debris by spinning for 10 min at 3000 *g*, 4°C, and was used fresh.

High-pressure liquid chromatography

The Merck–Hitachi LaChrom high-pressure liquid chromatography (HPLC) system (Hitachi Ltd, Tokyo, Japan) used included an L-7250 autosampler, an L-7420 ultraviolet detector, an L-7100 pump and a 250 × 4.6 mm C_{18} column (5 µm particle diameter), and was equipped with the Merck–Hitachi Chromatography Data Station software. dA and dA–CLB, and 2CdA and 2CdA–CLB were detected at $\lambda = 264$ nm and $\lambda = 256$ nm, respectively. For ester hydrolysis study, an isocratic method was employed using a 20-µl injection volume, a flow rate of 1 ml/min and a mobile phase of 50 mmol/l phosphate buffer pH 4.5 supplemented with 12% (v/v) acetonitrile for 2CdA–CLB or with 6% acetonitrile (v/v) for dA–CLB. Calibration was done using dA and 2CdA solutions in DMSO/0.1 mol/l phosphate buffer pH 7.4 (1 : 99, v/v).

Cell culture

All human leukemic cell lines were obtained from the American Type Culture Collection (Rockville, Maryland, USA). The cells were cultured at 37°C in a humidified 5% CO_2/95% air atmosphere, using RPMI-1640 medium supplemented with heat-inactivated (+ 56°C, 30 min) fetal calf serum (10%, v/v), penicillin (100 U/ml), streptomycin (0.1 mg/ml) and amphotericin B (25 µg/ml).

Hydrolysis studies

For the study of spontaneous hydrolysis, 10 mmol/l stock ester solutions in DMSO were diluted 1 : 99 with 0.1 mol/l phosphate buffer pH 7.4 and kept at + 37°C in a water bath. For the enzymatic hydrolysis study, 10-µl aliquots of the stock solutions were mixed with 980-µl aliquots of 0.1 mol/l phosphate buffer pH 7.4 and brought up to + 37°C (water bath). At time 0, 10 µl of PBMC lysate or porcine liver esterase suspension (5×10^{-2} U/ml) was added to each sample, and the mixtures were briefly vortexed and incubated at + 37°C with gentle mixing every 10 min. At scheduled time points, 25-µl aliquots of the mixtures were taken for HPLC analysis. In some experiments, the ester stock solutions (10 µl) were incubated with undiluted human blood plasma (990 µl), in which case the mixtures were deproteinized before HPLC analysis. Briefly, mixture aliquots were mixed with half their volume of 76 mmol/l uranyl acetate, vortexed and spun at 3000 *g* for 10 min. Next, 0.1 mol/l phosphate buffer pH 7.0 was added to the samples (1 : 5, v/v), the samples were gently vortexed and centrifuged as above, and the final supernatants were used for the analysis. To prevent degradation of dA and possibly dA–CLB by adenine deaminase, all reaction mixtures containing these compounds were supplemented with erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (final concentration: 25 µmol/l) and incubated for 30 min at + 37°C before adding blood plasma or lymphocyte lysate.

Cytotoxicity assay

Freshly harvested cultured leukemic cells were resuspended in the culture medium at 313 000, 254 000, or 188 000 cells/ml, for 48, 72 and 96 h cultures, respectively, seeded in 96-well plates (Nunc, Roskilde, Denmark) using 80 µl aliquots, and incubated at + 37°C in a humidified 5% CO_2/95% air atmosphere. One hour later, triplicate 20-µl aliquots of the medium supplemented with 0.5% (v/v) of DMSO (for control cultures) or triplicate 20-µl serial dilutions of the tested substances in the vehicle were added to the wells to give a 0–40 µmol/l concentration range and the cells were incubated further as above. The viable cell fraction was assessed using routine trypan blue exclusion test or a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay [23].

Uptake study

Cells used for the uptake study were suspended in the culture medium at 1.2–1.5 × 10^6 cells/ml and incubated at + 37°C in a water bath unless indicated otherwise. To prevent degradation of [8-^{14}C]dA or dA–CLB by adenine deaminase in lymphoid cells, the respective suspensions were preincubated for 30 min with 25 µmol/l erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride before adding the nucleoside or ester.

For assessing 2CdA uptake kinetics, 0.015 µCi (0.555 kBq, 10 µl) of [8-^{14}C]2CdA was added per milliliter of the final cell suspension at time 0. In parallel, a blank incubation with no cells present was run to enable correction for nonspecific binding. One-milliliter aliquots of the incubation mixtures were taken at scheduled time points and nonbound ^{14}C radioactivity was removed from the samples by filtration through Whatman GF/B filters in a Millipore harvester. The filters were then washed with 15 ml of ice-cold phosphate-buffered saline (pH 7.4) each, transferred to scintillation vials and treated with 200 µl of NSC II-Tissue Solubilizer. Fifteen minutes later 10 ml of Bray's scintillation fluid was added to each sample and the radioactivity of the samples measured 12 h later in a liquid scintillation counter.

As radiolabeled dA–CLB and 2CdA–CLB were unavailable, the uptake of the esters was studied indirectly using the following experimental designs.

Preincubation

Triplicate 1-ml cell suspension aliquots were incubated with the tested ester (1 µmol/l) for 10 min; the cells were then washed thrice by adding 1 ml of ice-cold culture medium, gentle vortexing, spinning at 800 *g* for 5 min and discarding the supernatant. The washed cells were resuspended in 1 ml of the medium containing 0.015 µCi of [8-^{14}C]dA (268 nmol/l), incubated for 30 min, collected and washed again, and the cell-bound

radioactivity was measured and corrected for nonspecific binding as for the uptake kinetics study.

Competition

Triplicate 1-ml cell suspension aliquots were incubated for 40 min with 0.015 μCi of [8-^{14}C]2CdA (273 nmol/l) or [8-^{14}C]dA (268 nmol/l) in the presence and absence of excess (2 μmol/l) of the respective ester or its parental compounds (singly or in equimolar concentrations). The cells were then collected and washed, and the cell-bound radioactivity was measured and corrected for nonspecific binding as above.

Acute toxicity study

Female BALB/c mice, 11 weeks of age, 18.6–19.6 g body weight (b.w.), were given a single intraperitoneal dose of dA–CLB (20 μl injection volume), CLB (20 μl) or the drug vehicle DMSO (20 μl). Seven mice were used in the vehicle-treated group (controls) and five mice were used for each drug dose tested. All mice were given free access to tap water and standard laboratory rodent chow, except that only water was allowed 19 h just before injections. On the injection day, the mice were subjected to a visual checkup 10 and 30 min after the injection, and then every hour. For the remainder of 14-day follow-up period the checkup was performed twice a day.

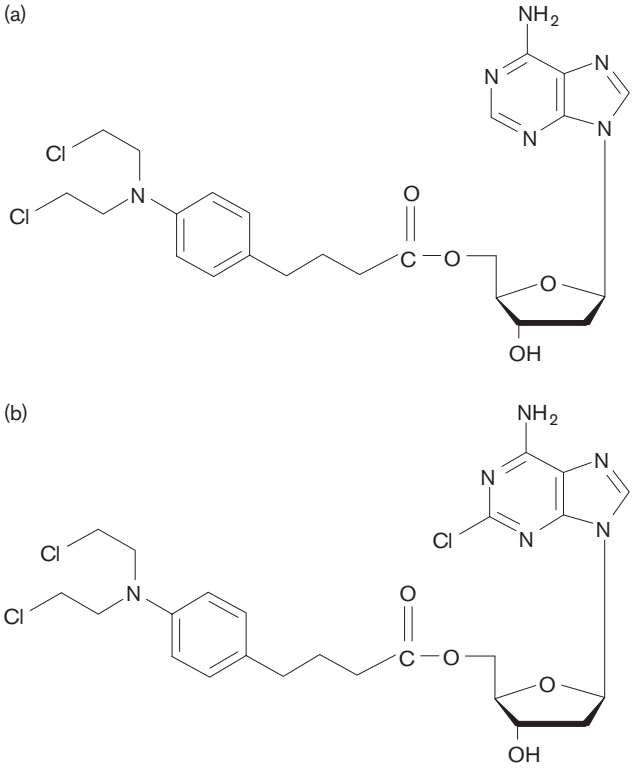
Statistical analysis

The significance of differences between individual means was tested by one-way analysis of variance followed by Dunnett's test or by two-way analysis of variance followed by Student–Neuman–Keuls test; in all cases, $P < 0.05$ was considered significant. LD_{50} values were calculated using logarithmic–probit regression. IC_{80} values were calculated on the basis of dose–response curves. All statistical analyses were done using the Statistica for Windows software version 6.0 (StatSoft, Tulsa, Oklahoma, USA).

Results

The Mitsunobu reaction used for nucleoside esterification results in selective acylation of the 5′-OH group [24]. Chemical structures of the newly obtained 5′-*O*-esters are shown in Fig. 1.

Fig. 1



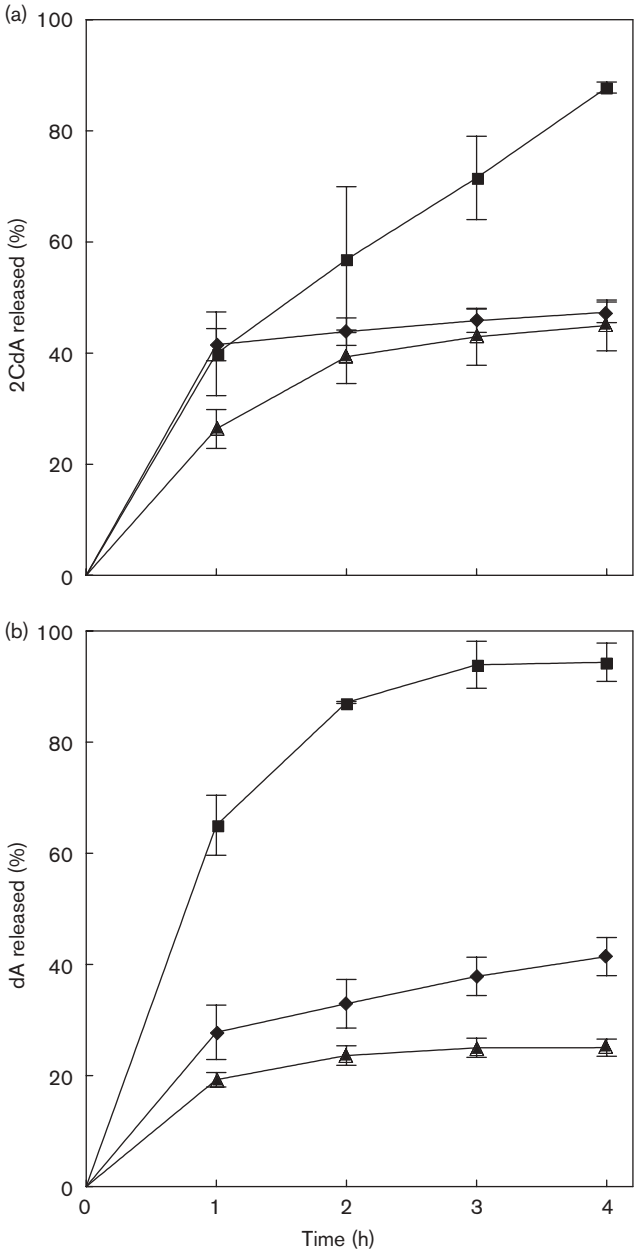
Chemical structures of 5′-*O*-esters of 2′-deoxyadenosine (a) and 2-chloro-2′-deoxyadenosine (b) with chlorambucil.

The 5′-*O*-ester bond showed no spontaneous hydrolysis at pH 7.4 after 24 h incubation at + 37°C (not shown). Porcine liver esterase was releasing the parental nucleoside faster from dA–CLB than from 2CdA–CLB; after 4 h incubation at + 37°C, free dA and 2CdA in the reaction mixtures corresponded, respectively, to 94.0 ± 0.8 and 87.6 ± 0.2%, (mean ± SD, $N = 4$) of the initial ester concentration. Nucleoside release slowed down markedly after less than 1 h incubation of the esters with human blood plasma or PBMC lysate, with a clear tendency for plateauing at free nucleoside concentration corresponding to or less than 50% of the initial ester concentration (Fig. 2).

Both 2CdA and 2CdA-CLB at 50 nmol/l or above concentration significantly reduced the relative survival of MOLT4 cells, whereas CLB and dA–CLB reduced the survival only at micromolar concentrations, and dA at upto 5 μmol/l concentration showed no toxicity to the cells. On the other hand, the toxicity of 2CdA–CLB did not exceed that of 2CdA (with or without CLB added, Fig. 3a and b) in MOLT4 cells, whereas dA–CLB was clearly more toxic to the cells than CLB (Fig. 3c and d). The latter finding was confirmed in a variety of human leukemic cell lines; depending on line used and culture duration, dA–CLB IC_{80} was 1.4–4.5-fold lower than CLB IC_{80} (Table 1). In general, the ester showed higher activity against lymphoblastoid leukemia cell lines than against other leukemic cell lines, which was also the case for unesterified CLB.

The kinetics of [8-^{14}C]2CdA uptake by human cells *in vitro* is shown in Fig. 4. Both PBMC and HL60 cells rapidly accumulated ^{14}C radioactivity for about 45 min; the rate of the process was about 4-fold higher in the leukemic cells. Later on, ^{14}C radioactivity

Fig. 2



Kinetics of nucleoside release from the 5′-*O*-esters of 2-chloro-2′-deoxyadenosine [2CdA (a)] and 2′-deoxyadenosine [dA (b)] with chlorambucil by porcine liver esterase (squares), human blood plasma (diamonds), and normal human peripheral blood mononuclear cells lysate (triangles). The results shown are mean percentage ± SD ($N = 4$) of the initial ester content.

accumulated at a markedly reduced steady rate that did not differ between PBMC and HL60 cells. As shown in Fig. 5(a and b), [8-^{14}C]dA uptake was over 2-fold lower in HL60 than in MOLT4 cells. Ten minutes preincubation with dA–CLB or its parental compounds showed no considerable effect on [8-^{14}C]dA uptake by HL60 cells (Fig. 5a), whereas it significantly reduced [8-^{14}C]dA uptake by MOLT4 cells (Fig. 5b).

The excess of either nonlabeled ester or its parental nucleoside markedly reduced the uptake of the respective radioactive nucleoside in human PBMC and leukemic cell lines. In MOLT4 cells, dA–CLB competed slightly, but significantly better than dA for the uptake, and a similar borderline significant effect ($P = 0.050$) was apparent for 2CdA–CLB (Fig. 5c and d).

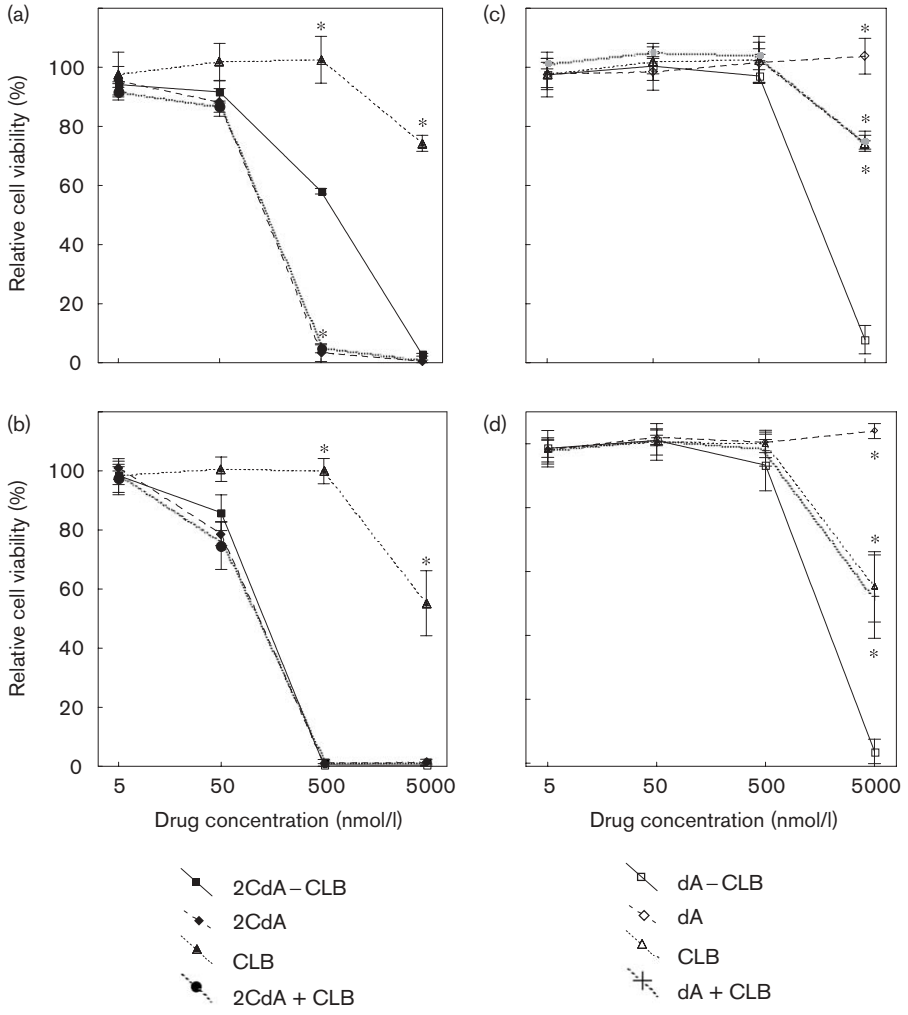
Mice injected with the lowest CLB dose (31.3 mg/kg b.w.) showed reduced mobility and 'depression' (a gloomish look) during the first postinjection day only, but no other side-effects, and all of them survived the 14-day follow-up. The same symptoms were apparent in all mice injected with 50.0 mg/kg b.w. of CLB. In this group, one mouse also showed tremors and died on the first postinjection day and three more mice died one day later. The last mouse showed normal mobility on the second day and dissolution of the depression three days later, and then remained asymptomatic till the end of the follow-up. All five mice administered with the highest CLB dose (80.0 mg/kg b.w.) showed depression, developed seizures and died during the first postinjection day. Four out of six surviving CLB-treated mice gained weight, whereas the remaining mice showed weight loss during the follow-up. CLB LD_{50} was 48.3 mg/kg b.w. (159 μmol/kg, 95% confidence limits: 152.7–165.6).

Mice injected with the lowest dose of dA–CLB (48.8 mg/kg b.w.) showed no side-effect but depression that resolved entirely during the first postinjection day, and all survived the 14-day follow-up period. Mice injected with the medium dA–CLB dose (78.1 mg/kg b.w.) showed depression and a decreased mobility that both dissipated during the first postinjection day in four mice. The four mice stayed asymptomatic for the rest of the follow-up, whereas the fifth mouse died on the second postinjection day. The highest dA–CLB dose (125 mg/kg b.w.) caused depression and reduced mobility in all five mice, four of which died on the second postinjection day; the remaining mouse fully recovered on the third postinjection day and remained asymptomatic for the rest of the follow-up. All surviving mice showed weight gain during the follow-up. dA–CLB LD_{50} was 100.8 mg/kg b.w. (188 μmol/kg, 95% confidence limits: 170.0–208.4).

Discussion

Therapeutic efficacy of cytostatics is usually limited by the damage they cause to proliferating normal cells such as those in bone marrow and gut epithelia. Thus, improving drug specificity and minimizing unwanted side-effects is of considerable importance in modern chemotherapy. One of the solutions relies upon using pharmacologically inactive drug precursors, or prodrugs,

Fig. 3



Relative viability of MOLT4 cells incubated for 48 (panels a and c) or 96 h (panels b and d) with 2-chloro-2′-deoxyadenosine–chlorambucil (2CdA–CLB) or its parental compounds (singly or in an equimolar mixture, panels a and b), or with 2′-deoxyadenosine–chlorambucil (dA–CLB) or its parental compounds (panels c and d). The results shown are mean percentage ± SD of the respective control value (for dimethyl sulfoxide-treated cell cultures) from four separate experiments; $*P < 0.01$ versus the respective ester-treated cultures, Dunnett's test.

which undergo conversion to active agents at or close to their intended site of action [1].

Most of the contemporary anticancer chemotherapies involve a concerted use of a number of cytostatics. This strategy is aimed at improving efficacy by reducing the chances for the development of drug resistance, whereas decreasing dosage and thus also side-effects of individual drugs. Secondary to this concept is the tactic utilized in this study, i.e. merging two agents in a single chemical entity (prodrug) with the aim to avoid summing-up of side-effects from a simple combination of the drugs.

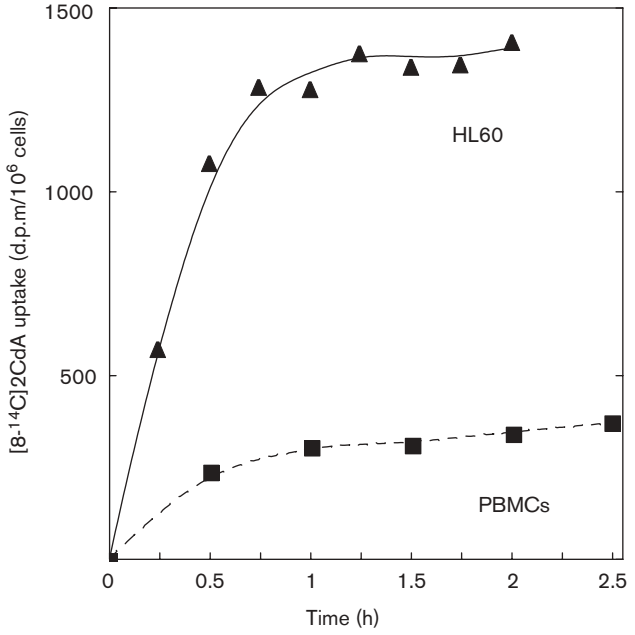
There are a number of reports on bi-functional CLB derivatives that have been synthesized in the search for CLB prodrugs. One example is CLB esters of anthraquinone derivatives, which were tested for their antitumor activity in mice bearing S-180 sarcoma ascitic cells or L1210 leukemic cells. Two of the esters appeared more active than unesterified CLB [25]. Antitumor activities of several CLB–paclitaxel conjugates were also studied, and one of these derivatives showed higher efficacy than its parent compounds (used either singly or as an equimolar mixture) both in the paclitaxel- and CLB-sensitive M109 and in the paclitaxel-resistant and CLB-sensitive M109/taxlR Madison murine lung carcinoma models [26]. Modification of CLB molecule by conjugating it with a DNA minor groove-binding carrier, such as the natural antibiotics distamycin and netropsin, was found not to considerably improve its activity in the human breast cancer cell line MCF-7 [27]. An amidine CLB derivative was, however, found to be a stronger inhibitor of protein

Table 1 Cytotoxicity of CLB and dA–CLB in human leukemic cell lines

Cell line	Incubation time (h)	IC_{80} (μmol/l)	
		CLB	dA–CLB
BALL[a]	72	8.0	4.5
CEM[a]	72	10.0	7.1
Jok-1[a]	72	25.0	8.3
KG-1[a]	72	27.0	9.8
MOLT3[a]	72	7.0	2.3
MOLT4[b]	48	6.6	2.4
MOLT4[b]	96	6.1	1.5
HL60[b]	48	37.8	11.5
HL60[b]	96	10.3	2.3
K562[b]	96	46.0[c]	17.2

CLB, chlorambucil; dA–CLB, 2′-deoxyadenosine–chlorambucil; BALL, B-cell acute lymphoblastoid leukemia; CEM, MOLT-3 and MOLT4, T-cell acute lymphoblastoid leukemias; KG-1, acute myeloid leukemia; HL60, acute promyelocytic leukemia; K562, chronic myeloid leukemia; Jok-1, hairy cell leukemia.
[a]Cell viability was assessed by trypan blue exclusion test.
[b]Cell viability was assessed by a modified MTT test.
[c]Extrapolated value.

Fig. 4



Uptake of 8-^{14}C-labeled 2-chloro-2′-deoxyadenosine ([8-^{14}C]2CdA) by normal human peripheral blood mononuclear cells (PBMCs) and acute promyelocytic leukemia HL60 cells.

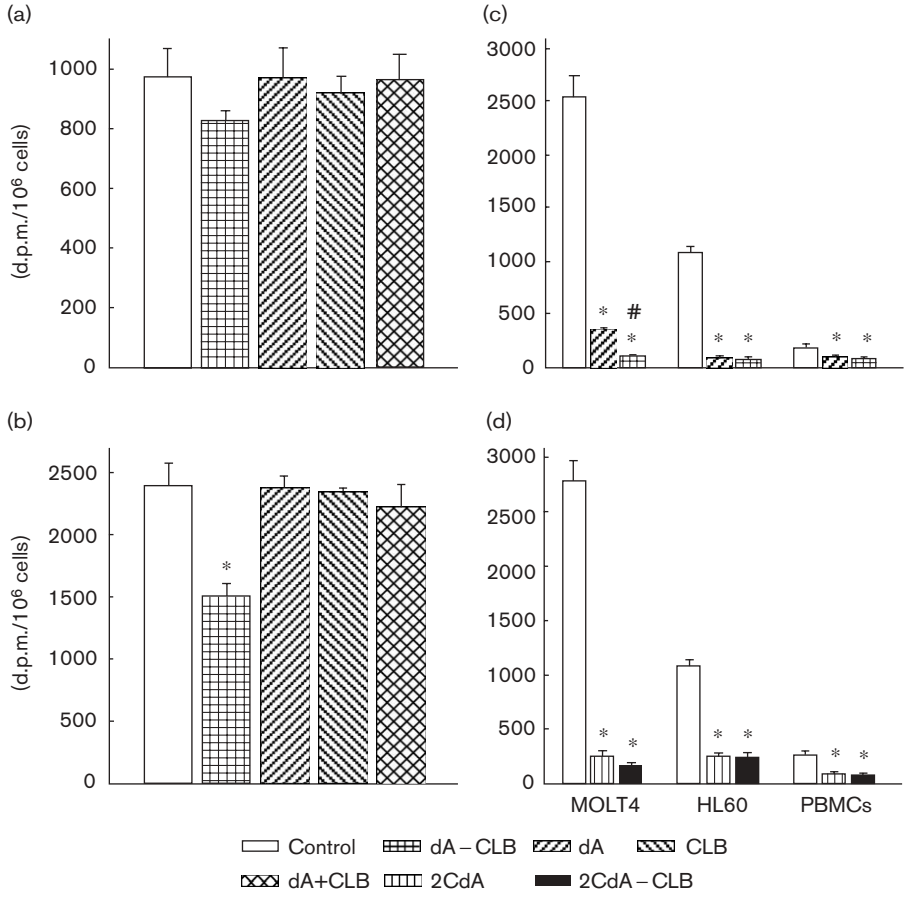
and DNA synthesis in MCF-7 cells [28]. Recently, an ester of the antiangiogenic drug combretastatin and CLB has been obtained that was considerably more potent *in vitro* than a plain mixture of its parental compounds [29].

Bi-functional candidate prodrugs of CLB studied also included a number of steroidal derivatives. The steroid part of such molecule is not a simple biological carrier, but also affects the ability of the nitrogen mustard to interact with different sequences of bases in the minor or major groove of DNA. The propensity for enhancing or decreasing antileukemic activity of alkylating agent, which depends on the chemical structure of steroid, was recently intensively investigated [30–33]. It was found that some esters in which aromatic nitrogen mustard was linked to a modified steroidal moiety showed enhanced antileukemic activity and reduced toxicity in comparison with those of nonderivatized nitrogen mustards or their conjugates with a simple steroidal skeleton. So far, the only US Food and Drug Administration-approved steroidal derivative of CLB is the prednisolone ester prednimustine that is used in patients with hematological malignancies [34].

The inspiration for our study on the 5′-*O*-esters of 2CdA and dA with CLB was the reports on synergistic cell kill in human chronic lymphoblastoid leukemia cells *in vitro* caused by combined use of 2CdA and CLB [8,9]; this effect might be related to distinct mechanisms of action of the drugs. Phosphorylation of 2CdA by dCK followed by nonspecific nucleotide kinases results in intracellular accumulation of cladribine di- and triphosphates. 2CdA–diphosphate inhibits ribonucleotide reductase, and 2CdA–triphosphate inhibits DNA polymerases involved in genomic DNA replication and repair [35]. 2CdA–triphosphate can also be incorporated into newly synthesized DNA, causing chain termination. These phenomena result in accumulation of DNA breaks and initiation of apoptosis [36]. Cytotoxicity of CLB relies mostly on its covalent binding to DNA (preferably to N^7 of guanine residues), consequential DNA cross-linking, misreading the genetic code, and generation of single- and double-strand DNA breaks as well. CLB shows also a propensity for covalent binding to RNA and proteins, which likely contributes to its cytotoxicity [37].

Disappointingly, 2CdA–CLB showed somewhat poorer cytotoxicity than 2CdA alone or in an equimolar combination with CLB in leukemic cell cultures in this study (Fig. 3a and b), suggesting no potential therapeutic gain from merging the two cytostatics into a dual prodrug. This was unlikely to result from relative stability and possible lack of or low toxicity of 2CdA–CLB *per se*, as the ester bond was susceptible to the hydrolytic action of PBMC lysate and human blood plasma (Fig. 2a). Hydrolysis of this bond is critical for 2CdA ester cytotoxicity, as esterase-resistant 5′-*O*-esters of 2CdA appear inactive against malignant cells [38]. On the other hand, an overly rapid hydrolysis, e.g. by liver esterases that are primarily responsible for *in-vivo* hydrolysis of ester prodrugs [20,39] or by the esterases present in blood plasma, may extinguish any potential therapeutic advantage by decomposing a candidate ester prodrug before its reaches the target cells.

Fig. 5



Uptake of [8-^{14}C]nucleoside by normal human peripheral blood mononuclear cells (PBMCs) and leukemic cells. Left panels show the effect of preincubation with 2′-deoxyadenosine–chlorambucil dA–CLB or its parental compounds (singly or in an equimolar mixture) on subsequent [8-^{14}C]dA uptake [(a) HL60 cells, one-way analysis of variance: F (4,10) = 1.7, P = 0.23; (b) MOLT4 cells, F (4,10) = 26.2, P = 0.003); the results shown are mean ± SD, N = 4. *P < 0.01 versus the vehicle-preincubated cells, Dunnett's test]. Right panels illustrate the effect of about 8-fold excess of the respective ester or free nucleoside on the uptake of [8-^{14}C]dA panel (c); and [8-^{14}C]2CdA panel (d). Two-way analysis of variance yielded significant effects of cell line, competitor type (free nucleoside or ester) and competitor type × cell line interaction on [^{14}C]nucleoside uptake [(c) F (2,18) = 348.5, $P < 10^{-6}$, F (2,18) = 748.8, $P < 10^{-6}$, and F (4,18) = 240.0, $P < 10^{-6}$, respectively; (d) F (2,18) = 335.5, $P < 10^{-6}$, F (2,18) = 853.3, $P < 10^{-6}$, and F (4,18) = 261.1, $P < 10^{-6}$, respectively]. The results shown are mean ± SD, N = 4; *P < 0.01 versus the cells incubated with the drug vehicle, $^{\#}P$ < 0.05 versus the cells incubated with the excess of the respective nonlabeled nucleoside, Student–Neuman–Keuls' test.

Interestingly, hydrolysis rates of the studied esters decreased considerably after 1 h incubation with blood plasma or PBMC lysate, when free nucleoside present in the reaction mixtures corresponded to less than 50% of the ester amount added (Fig. 2a and b). These results suggest inactivation of the esterases present in the reaction mixtures owing to their alkylation by the CLB moiety of the ester molecule or by CLB released from the ester; this would imply useless depletion of the prodrug or drug. No similar change in hydrolysis rate was, however, observed using porcine liver esterase in the absence of other proteins in the reaction mixture. Therefore, a more likely explanation is futile 'consumption' of the ester owing to its CLB moiety alkylating some components of the lysate or serum [40], and hence rendering the 5′-*O*-ester bond less accessible or inaccessible for esterases. It has also been shown that breakdown of CLB and its major active metabolite, phenylacetic acid mustard, was hampered by human serum albumin [41]. This suggests that CLB and some of its derivatives can form noncovalent protein adducts representing a 'biodepot' form of the drugs; this process might protect the drugs from rapid enzymatic or nonenzymatic alteration. It is, however, not known if the CLB esters studied can form such adducts.

In vitro studies employing sequential exposure to CLB and 2CdA showed synergy of the drugs in lymphocytes

from CLL patients when 2CdA treatment followed CLB treatment [8]. On the other hand, simultaneous exposure of CLL patient lymphocytes to CLB and 2CdA showed synergic or additive effects in 51–61% of the drug combinations and antagonistic effects in the remaining combinations [9]. In human-derived B-cell lymphoma cell lines the combination of 2CdA and CLB was inferior to either agent used alone [10]. In our study, 2CdA–CLB was not superior to 2CdA alone or in an equimolar mixture with CLB. Most likely it was due to the fact that the amount of CLB generated from the ester was too small to significantly add to the cytotoxic effect of 2CdA released from the conjugate (see Fig. 3a and b).

Noteworthy, the dA–CLB ester that was included in this study primarily as a model compound showed some promise as a potential prodrug, as it appeared more active against a variety of human lymphoid and myeloid malignant cell lines than its parental CLB. This could be due to the presence of the dA moiety, which likely enabled transmembrane influx of the ester via nucleoside transporters and thus promoted its intracellular conversion to CLB.

[8-^{14}C]dA and [8-^{14}C]2CdA uptake by MOLT4 cells greatly exceeded that by HL60 cells (Fig. 5) despite the fact that the latter are considerably larger (15–20 versus 8–12 μm diameter). The possible reason was the lymphoidal origin of MOLT4 cells, which is associated with much higher dCK activity [42] and, probably, also more rapid nucleoside catabolism. This may result in higher dA–CLB utilization and toxicity to the cells. dA–CLB markedly reduced [8-^{14}C]dA uptake by MOLT4 and HL60 cells and normal human PBMCs when the cells were exposed to the ester and the [^{14}C]nucleoside simultaneously or in succession (Fig. 5a–c). One may argue that this was due to the ester's CLB moiety alkylating and thus blocking nucleoside transporters. Lymphoid cells, however, synthesize dA *de novo* [43]. Hence, it is unlikely that the dA–CLB-related reduction in cell survival resulted from disturbed influx of the nucleoside; the more plausible reason was the aforementioned enhanced transmembrane delivery of CLB in the form of the ester.

A number of reports on candidate CLB prodrugs containing a carrier moiety, e.g. fatty acid [44], sugar [45], proline [46,47], spermidine [48] and cyclic peptide [49] CLB derivatives, are found. Yet, the antitumor activity *in vitro* of these compounds did not translate into a useful medication. In some cases that was due to increased neurotoxicity of the conjugate in comparison to those of unconjugated CLB [48]. Interestingly, dA–CLB acute toxicity in mice, including neurotoxicity, was apparently lower than that of free CLB. This fact in conjunction with cytotoxicity data acquired in a number of human leukemic cell lines shows the promise for usability of the ester in a similar spectrum of oncohematological diseases, but with a better therapeutic index than that of CLB. Therefore, an *in vivo* study of antileukemic efficacy (e.g. in a rodent model) of the 5′-*O*-ester dA–CLB seems warranted.

References

1 Rao HSP. Capping drugs: development of prodrugs. *Resonance* 2003; 19–27. Internet publication: http://www.ias.ac.in/resonance/Feb2003/pdf/Feb2003p19-27.pdf.

2 Food and Drug Administration. Product information.Website:http://www.accessdata.fda.gov/scripts/cder/onctools/labels.cfm?GN=chlorambucil.

3 Kawasaki H, Carrera CJ, Piro LD, Saven A, Kipps TJ, Carson DA. Relationship of deoxycytidine kinase and cytoplasmic 5′-nucleotidase to the chemotherapeutic efficacy of 2-chlorodeoxyadenosine. *Blood* 1993; **81**:597–601.

4 Robak T, Blasinska-Morawiec M, Blonski J, Hellmann A, Halaburda K, Konopka L, *et al.* 2-chlorodeoxyadenosine (cladribine) in the treatment of hairy cell leukemia and hairy cell leukemia variant: 7-year experience in Poland. *Eur J Haematol* 1999; **62**:49–56.

5 Robak T, Gora-Tybor J, Krykowski E, Walewski JA, Borawska A, Pluzanska A, *et al.* Activity of 2-chlorodeoxyadenosine (Cladribine) in 2-hour intravenous infusion in 94 previously treated patients with low grade non-Hodgkin's lymphoma. *Leuk Lymphoma* 1997; **26**:99–105.

6 Robak T. New agents in chronic lymphocytic leukemia. *Curr Treat Options Oncol* 2006; **7**:200–212.

7 Johnson SA, Birchall J, Luckie C, Oscier DG, Owen RG. Guidelines on the management of Waldenstrom macroglobulinaemia. *Br J Haematol* 2006; **132**:683–697.

8 Begleiter A, Wang H, Verburg L, Lee K, Israels LG, Mowat MR, *et al. In vitro* cytotoxicity of 2-chlorodeoxyadenosine and chlorambucil in chronic lymphocytic leukemia. *Leukemia* 1996; **10**:1959–1965.

9 Callea I, Console G, Sculli G, Filangeri M, Messina G, Morabito F. Chlorambucil synergizes with purine analogs in inducing *in vitro* cytotoxicity in B-cell chronic lymphocytic leukemia. *Haematologica* 1998; **83**:756–757.

10 Hoffman M, Xu JC, Lesser M, Rai K. Cytotoxicity of 2-chlorodeoxadenosine (cladribine, 2-cdA) in combination with other chemotherapy drugs against two lymphoma cell lines. *Leuk Lymphoma* 1999; **33**:141–145.

11 Laurencet FM, Zulian GB, Guetty-Alberto M, Iten PA, Betticher DC, Alberto P. Cladribine with cyclophosphamide and prednisone in the management of low-grade lymphoproliferative malignancies. *Br J Cancer* 1999; **79**:1215–1219.

12 Montillo M, Tedeschi A, O'Brien S, Di Raimondo F, Lerner S, Ferrajoli A, *et al.* Phase II study of cladribine and cyclophosphamide in patients with chronic lymphocytic leukemia and prolymphocytic leukemia. *Cancer* 2003; **97**:114–120.

13 Robak T, Blonski JZ, Kasznicki M, Gora-Tybor I, Dwilewicz-Trojaczek J, Boguradzki P, *et al.* Cladribine combined with cyclophosphamide and mitoxantrone as front-line therapy in chronic lymphocytic leukemia. *Leukemia* 2001; **15**:1510–1516.

14 Robak T, Blonski JZ, Kasznicki M, Gora-Tybor J, Dwilewicz-Trojaczek J, Stella-Holowiecka B, *et al.* Cladribine combined with cyclophosphamide is highly effective in the treatment of chronic lymphocytic leukemia. *Hematol J* 2002; **3**:244–250.

15 Tefferi A, Witzig TE, Reid JM, Li CY, Ames MM. Phase I study of combined 2-chlorodeoxyadenosine and chlorambucil in chronic lymphoid leukemia and low-grade lymphoma. *J Clin Oncol* 1994; **12**:569–574.

16 Tefferi A, Levitt R, Li CY, Schroeder G, Tschetter LK, Michalak JC, *et al.* Phase II study of 2-chlorodeoxyadenosine in combination with chlorambucil in previously untreated B-cell chronic lymphocytic leukemia. *Am J Clin Oncol* 1999; **22**:509–516.

17 Weber DM, Dimopoulos MA, Delasalle K, Rankin K, Gavino M, Alexanian R. 2-Chlorodeoxyadenosine alone and in combination for previously untreated Waldenstrom's macroglobulinemia. *Semin Oncol* 2003; **30**:243–247.

18 Tefferi A, Li CY, Reeder CB, Geyer SM, Allmer C, Levitt R, *et al.* A phase II study of sequential combination chemotherapy with cyclophosphamide, prednisone, and 2-chlorodeoxyadenosine in previously untreated patients with chronic lymphocytic leukemia. *Leukemia* 2001; **15**:1171–1175.

19 Robak T, Blonski JZ, Gora-Tybor J, Jamroziak K, Dwilewicz-Trojaczek J, Tomaszewska A, *et al.* Cladribine alone and in combination with cyclophosphamide or cyclophosphamide plus mitoxantrone in the treatment

of progressive chronic lymphocytic leukemia: report of a prospective, multicenter, randomized trial of the Polish Adult Leukemia Group (PALG CLL2). *Blood* 2006; **108**:473–479.
20 Satoh T, Taylor P, Bosron WF, Sanghani SP, Hosokawa M, La Du BN. Current progress on esterases: from molecular structure to function. *Drug Metab Dispos* 2002; **30**:488–493.
21 Xu G, Zhang W, Ma MK, McLeod HL. Human carboxylesterase 2 is commonly expressed in tumor tissue and is correlated with activation of irinotecan. *Clin Cancer Res* 2002; **8**:2605–2611.
22 Adair CG, McElnay JC. Studies on the mechanism of gastrointestinal absorption of melphalan and chlorambucil. *Cancer Chemother Pharmacol* 1986; **17**:95–98.
23 Tada H, Shiho O, Kuroshima K, Koyama M, Tsukamoto K. An improved colorimetric assay for interleukin 2. *J Immunol Methods* 1986; **93**:157–165.
24 Mitsunobu O. The use of diethyl azodicarboxylate and triphenylphosphine in synthesis and transformation of natural products. *Synthesis* 1981; :1–28.
25 Jin GZ, You YJ, Kim Y, Nam NH, Ahn BZ. Esters of chlorambucil with 2-substituted 1,4-dihydroxy-9,10-anthraquinones as multifunctional anticancer agents. *Eur J Med Chem* 2001; **36**:361–366.
26 Wittman MD, Kadow JF, Vyas DM, Lee FL, Rose WC, Long BH, *et al.* Synthesis and antitumor activity of novel paclitaxel–chlorambucil hybrids. *Bioorg Med Chem Lett* 2001; **11**:811–814.
27 Bartulewicz D, Bielawski K, Bielawska A, Rozanski A. Synthesis, molecular modelling, and antiproliferative and cytotoxic effects of carbocyclic derivatives of distamycin with chlorambucil moiety. *Eur J Med Chem* 2001; **36**:461–467.
28 Sienkiewicz P, Bielawski K, Bielawska A, Palka J. Amidine analogue of chlorambucil is a stronger inhibitor of protein and DNA synthesis in breast cancer MCF-7 cells than is the parent drug. *Eur J Pharmacol* 2004; **492**:95–101.
29 Coggiola B, Pagliai F, Allegrone G, Genazzani AA, Tron GC. Synthesis and biological activity of mustard derivatives of combretastatins. *Bioorg Med Chem Lett* 2005; **15**:3551–3554.
30 Fousteris MA, Koutsourea AI, Arsenou ES, Papageorgiou A, Mourelatos D, Nikolaropoulos SS. Structure–anti-leukemic activity relationship study of B- and D-ring modified and non-modified steroidal esters of chlorambucil. *Anticancer Drugs* 2006; **17**:511–519.
31 Papageorgiou A, Koutsourea AI, Arsenou ES, Fousteris MA, Mourelatos D, Nikolaropoulos SS. Structure–anti-leukemic activity relationship study of B- and D-ring modified and non-modified steroidal esters of chlorambucil's active metabolite. *Anticancer Drugs* 2005; **16**:1075–1082.
32 Arsenou ES, Fousteris MA, Koutsourea AI, Papageorgiou A, Karayianni V, Mioglou E, *et al.* The allylic 7-ketone at the steroidal skeleton is crucial for the antileukemic potency of chlorambucil's active metabolite steroidal esters. *Anticancer Drugs* 2004; **15**:983–990.
33 Karayianni V, Papageorgiou A, Mioglou E, Iakovidou Z, Mourelatos D, Fousteris M, *et al.* 7-Keto hybrid steroidal esters of nitrogen mustard: cytogenetic and antineoplastic effects. *Anticancer Drugs* 2002; **13**:637–643.
34 IARC. Prednimustine. *IARC Monogr Eval Carcinog Risks Hum* 1990; **50**:115–122.
35 Parker WB, Bapat AR, Shen JX, Townsend AJ, Cheng YC. Interaction of 2-halogenated dATP analogs (F, Cl, and Br) with human DNA polymerases, DNA primase, and ribonucleotide reductase. *Mol Pharmacol* 1988; **34**:485–491.
36 Bouchard VJ, Rouleau M, Poirier GG. PARP-1, a determinant of cell survival in response to DNA damage. *Exp Hematol* 2003; **31**:446–454.
37 Florea-Wang D, Haapala E, Mattinen J, Hakala K, Vilpo J, Hovinen J. Reactions of *N*,*N*-bis(2-chloroethyl)-*p*-aminophenylbutyric acid (chlorambucil) with 2′-deoxyadenosine. *Chem Res Toxicol* 2003; **16**:403–408.
38 Grieb P, Kryczka T, Wojtowicz R, Kawiak J, Kazimierczuk Z. 5′-Esters of 2′-deoxyadenosine and 2-chloro-2′-deoxyadenosine with cell differentiation-provoking agents. *Acta Biochim Pol* 2002; **49**:129–137.
39 Imai T, Yoshigae Y, Hosokawa M, Chiba K, Otagiri M. Evidence for the involvement of a pulmonary first-pass effect via carboxylesterase in the disposition of a propranolol ester derivative after intravenous administration. *J Pharmacol Exp Ther* 2003; **307**:1234–1242.
40 Noort D, Hulst AG, Jansen R. Covalent binding of nitrogen mustards to the cysteine-34 residue in human serum albumin. *Arch Toxicol* 2002; **76**:83–88.
41 Lof K, Hovinen J, Reinikainen P, Vilpo LM, Seppala E, Vilpo JA. Kinetics of chlorambucil *in vitro*: effects of fluid matrix, human gastric juice, plasma proteins and red cells. *Chem Biol Interact* 1997; **103**:187–198.
42 van der Wilt CL, Kroep JR, Loves WJ, Rots MG, Van Groeningen CJ, Kaspers GJ, *et al.* Expression of deoxycytidine kinase in leukaemic cells compared with solid tumour cell lines, liver metastases and normal liver. *Eur J Cancer* 2003; **39**:691–697.
43 Reichard P. Interactions between deoxyribonucleotide and DNA synthesis. *Annu Rev Biochem* 1988; **57**:349–374.
44 Anel A, Halmos T, Torres JM, Pineiro A, Antonakis K, Uriel J. Cytotoxicity of chlorambucil and chlorambucil–fatty acid conjugates against human lymphomas and normal human peripheral blood lymphocytes. *Biochem Pharmacol* 1990; **40**:1193–1200.
45 Iglesias-Guerra F, Candela JI, Bautista J, Alcudia F, Vega-Perez JM. Alkylating agents from sugars. Alkyl hexopyranoside derivatives as carrier systems for chlorambucil. *Carbohydr Res* 1999; **316**:71–84.
46 Bielawska A, Chrzanowski K, Bielawski K, Palka J. Decreased cytotoxicity and increased antimitotic activity of a proline analogue of chlorambucil as a prodrug susceptible to the action of fibroblast's prolidase. *Pharmazie* 2001; **56**:290–294.
47 Bielawska A, Bielawski K, Chrzanowski K, Wolczynski S. Prolidase-activated prodrug for cancer chemotherapy cytotoxic activity of proline analogue of chlorambucil in breast cancer MCF-7 cells. *Farmaco* 2000; **55**:736–741.
48 Verschoyle RD, Carthew P, Holley JL, Cullis P, Cohen GM. The comparative toxicity of chlorambucil and chlorambucil–spermidine conjugate to BALB/c mice. *Cancer Lett* 1994; **85**:217–222.
49 Sheh L, Chang HW, Ong CW, Chen SL, Bailly C, Linssen RC, *et al.* Synthesis, DNA binding, and sequence specificity of DNA alkylation by some novel cyclic peptide-chlorambucil conjugates. *Anticancer Drug Des* 1995; **10**:373–388.