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In Vivo Antitumor Activity of Clitocine, an Exocyclic Amino Nucleoside Isolated from *Lepista inversa*

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A biologically guided fractionation from Lepista inversa (Scop.: Fr.) led to the isolation of clitocine, an exocyclic amino nucleoside. This compound and two mixtures of β/α anomers (mixture A, 40:60 and mixture B, 80:20) were synthesized or isolated depending on the purification procedure. The β anomer and clitocine mixtures A and B showed similar cytotoxic activities with IC_{50} values ranging from 20.5 to 42 nM in murine cancer cell lines (3LL and L1210) and from 185 to 578 nM in human cancer cell lines (DU145, K-562, MCF7, and U251). An in vivo study of mixture B was carried out on 3LL- and L1210-tumor-bearing mice. Clitocine solubilized in β-hydroxypropylcyclodextrin and injected at concentrations of 0.5, 3, and 5 mg kg^{-1} did not significantly increase the survival rate and lifespan of 3LL-tumor-bearing mice. In contrast, clitocine showed antitumor activity on L1210-tumor-bearing mice with a significant increase in lifespan and a decrease in the development of ascites observed at 3 mg kg^{-1}. The induction of apoptosis may be the basis of the antitumor activity of clitocine against L1210 as suggested by flow-cytometry analysis of cells treated in vitro.

Introduction

Mushrooms are well-known to produce unusual classes of compounds with a wide variety of biological activities (including antimicrobial, antiviral, and antitumor properties) and are very attractive as a potential source of new therapeutic agents.[1] About 200 mushroom species produce compounds that markedly inhibit the growth of various tumor cells in vitro or in vivo.[2] During the course of our screening for cytotoxic metabolites from basidiomycetes (sporophores of higher mushrooms), we recently reported the cytotoxic evaluation of 80 species of Amanitales, Boletales, Polyporales, Russulales[3] and Tricholomatales[4] on two murine (3LL and L1210) and four human (DU145, K-562, MCF7, and U251) cancer cell lines. Among the Tricholomatales investigated, better scores were obtained, with three *Lepista* extracts exhibiting IC_{50} values < 20 μg mL^{-1}. We focused our study on *Lepista inversa* (Scop.: Fr.), a species belonging to the *Tricholomataceae* family[5] which showed cytotoxicity against various cancer cell lines with IC_{50} values in the range of 0.2–18.5 μg mL^{-1}. An in vivo study of the antitumor activity of the methanolic extract of *L. inversa* was carried out by using intraperitoneally transplanted lymphocytic leukemia (L1210) cells and intramuscularly transplanted Lewis lung carcinoma (3LL) cells in mice. At 75 mg kg^{-1}, the extract increased the lifespan of L1210- and 3LL-tumor-bearing mice by 50 and 14%, respectively.[6] Herein, we report the biologically guided isolation of clitocine, which was found to be the major active compound from the crude extract of *L. inversa*. Clitocine isolates from this mushroom have already been shown to exhibit strong insecticidal activity against the pink bollworm *Pectinophora gossypiella* and cytotoxic activities against some cancer cell lines (L1210, WI-L2, and CCRF-CEM).[7,8] We report additional data on cell lines 3LL, DU145, K-562, MCF7, and U251. An epimerization of clitocine was also observed, and was dependent on the purification procedure, as discussed herein. The total synthesis of clitocine allowed an in vivo study on the 3LL and L1210 mice models after overcoming difficulties with compound solubility. A flow-cytometry analysis of treated L1210 cells was also performed to investigate the basis of clitocine cytotoxicity.

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Results

Biologically guided fractionation and isolation of clitocine A

The crude extract I (152 g), afforded by methanolic extraction of *L. inversa* (5 kg), showed significant cytotoxicity against the 3LL and L1210 murine tumor-cell lines (IC_{50} 3LL: 2.30 μg mL^{-1}; IC_{50} L1210: 2.00 μg mL^{-1}; Table 1).

The first attempt to isolate the active compound consisted of two successive partitions of a MeOH extract with two solvents of increasing polarity (petroleum benzene and methyl ethyl ketone) to yield three fractions: a petroleum benzene soluble extract (PB), a methyl ethyl ketone soluble extract (MEK) and a residual extract (RE) (Figure 1). The active compound was concentrated in the methyl ethyl ketone soluble extract and had good activity against 3LL cells (IC_{50} = 0.32 μg mL^{-1}) and L1210 cells (IC_{50} = 0.27 μg mL^{-1}) (Table 1). A TLC analysis of these fractions indicated the presence of nonpolar compounds in the less active PB extract and the presence of polar compounds (such as amino acids) in the inactive residual extract. The fractionation of MEK extract by vacuum liquid chromatography (VLC) with a solvent mixture of increasing polarity (from CH_2Cl_2 to MeOH) afforded four fractions, A–D (Figure 1). Fraction D, the most active against 3LL and L1210 cells ($IC_{50} \approx 0.40$ μg mL^{-1}; Table 1), was separated on silica gel with a mixture of CH_2Cl_2/MeOH, subjected to circular centrifugal chromatography (CCC), followed by RP HPLC (MeOH/H_2O 20:80) to yield clitocine mixture A (11.5 mg; Figure 1). Adenosine (27.5 mg) and urea (80.3 mg) were also isolated from fraction D and identified by comparison with published spectroscopic data[9,10] or with the commercially available data for urea. The clitocine content of extract I and fractions MEK and D was determined by HPLC to be 0.75, 6.4, and 3.2%, respectively.

Table 1. Cytotoxicity of *L. inversa* extracts and fractions A–D toward tumor cells.[a]

Cell line	IC_{50} [μg mL^{-1}][a]							
	I[b]	PB[c]	MEK[d]	RE[e]	A	B	C	D
3LL	2.30 ± 1.0	27.3 ± 5.3	0.32 ± 0.1	14.5 ± 0.8	ND	ND	ND	0.42 ± 0.2
L1210	2.00 ± 0.5	14.7 ± 6.4	0.27 ± 0.1	11.5 ± 3.2	6.60 ± 3.3	13.2 ± 4.1	0.49 ± 0.3	0.37 ± 0.1

[a] Values reflect the mean ± SD of at least three repeat experiments. [b] Crude extract. [c] Petroleum benzene soluble extract. [d] Methyl ethyl ketone soluble extract. [e] Residual extract. ND: not determined.

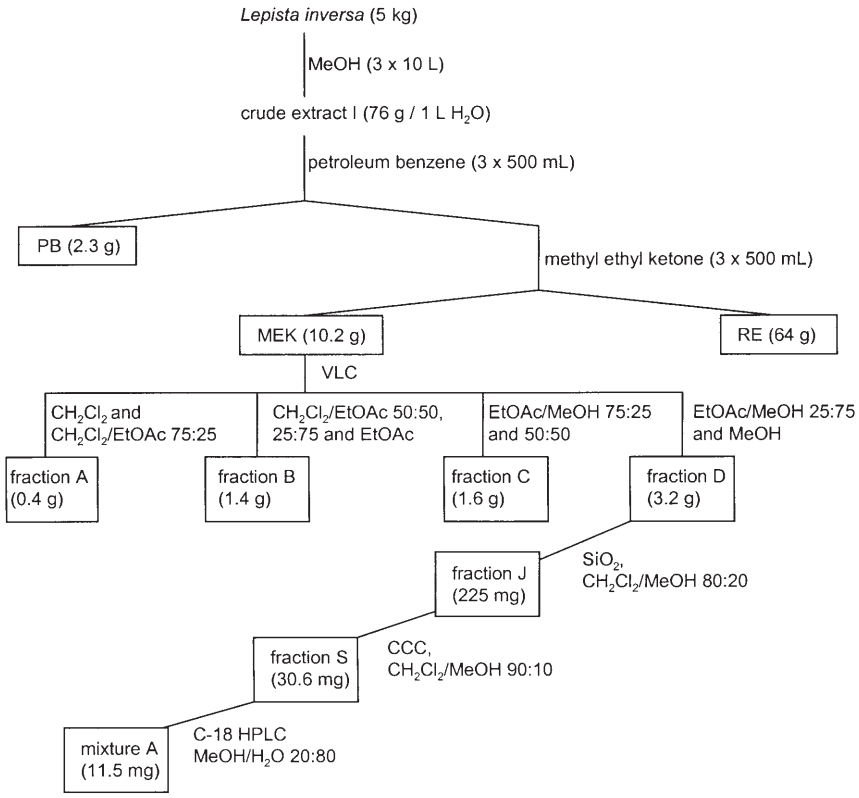

Figure 1. Isolation procedure for clitocine mixture A from *L. inversa*.

The second set was performed by using a procedure described previously.[7] A portion of crude extract I was extracted successively with *n*-hexane, $CHCl_3$, and EtOAc. The ethyl acetate soluble fraction (508 mg) was partitioned with droplet countercurrent chromatography (DCC) with $CHCl_3$/MeOH/H_2O 13:7:4. Column chromatography (C-18) with MeOH/H_2O 95:5 gave the clitocine β anomer (23 mg).

Clitocine, a pale-yellow amorphous powder (mp: 230–232 °C) with R_f = 0.55 (SiO_2, CH_2Cl_2/MeOH 80:20) has the molecular formula $C_9H_{13}N_5O_6$ as determined by high resolution ESI MS (HRESI MS). The IR spectrum indicated the presence of hydroxy (3332 cm^{-1}), amino (3438 cm^{-1}), and nitro (1520 cm^{-1}) groups and aromatic rings (1650 cm^{-1}) and was nearly identical to previously reported IR spectra.[7,8] The UV/Vis spectrum was similar to that previously described[8] and was characterized by absorption maxima at λ = 212 nm (ε = 22962) and λ = 332 nm (ε = 8611) with a shoulder at λ = 230 nm. The ^{1}H and ^{13}C NMR spectra indicated the presence of a ribose moiety and were identical to those reported earlier,[7,8] thus confirming the structure of clitocine (Figure 2). The anomeric purity of clitocine given by the β/α ratio was deter-

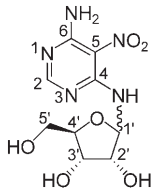

Figure 2. Structure of clitocine with the conventional atom numbering shown.

mined by integration of the anomeric proton. The ribose 1′ protons were assigned as double doublets at $\delta = 5.79$ ppm ($J = 7.8$ and 3.4 Hz) for the β anomer and at 6.03 ppm ($J = 8.5$ and 4.8 Hz) for the α anomer, respectively. The NH protons were assigned as doublets at 9.29 ($J = 7.8$ Hz) for the β anomer and at 9.96 ($J = 8.5$ Hz) for the α anomer, respectively. However, as these are exchangeable protons, they were not used for the determination of the anomeric ratio. This ratio was determined to be β/α 40:60 for the mixture A. In the ^{13}C NMR spectrum of this mixture, signals of each anomer α and β were assigned. The presence of intramolecular hydrogen bonding between the 4- and 6-amino hydrogen atoms ($\delta = 9.29$ and 8.57 ppm, respectively) and the oxygen atoms of the 5-nitro group of clitocine was confirmed by X-ray crystallography and NMR spectroscopy (through the de-shielded position of the NH signal).[8,11] The presence of these interactions could explain the low solubility of clitocine in water.

Clitocine was obtained as various mixtures of β and α anomers according to the process of isolation. Whereas the isolation of pure β anomer was reported for the use of sodium methoxide during the deprotection step followed by fractional recrystallization from methanol,[8] we obtained clitocine as the β/α 80:20 mixture B by using silica gel column chromatography during the last purification step. In contrast, pure β anomer was isolated from extract I with DCC and C-18 column chromatography as previously described.[7] Clitocine anomerization was examined under various conditions: adsorption on SiO_2 with CH_2Cl_2/MeOH 80:20 or MeOH for 30 min or 24 h, or with H_2O at 37 °C for 48 h. Dried β anomer appeared to be stable over a period of 48 h, whereas ≈15% converted into the α anomer in aqueous solution at 37 °C or with silica gel. An increase in formation of the α anomer to 30% was also observed by treatment of mixture B (β/α 80:20) in aqueous conditions. However, mixture A (β/α 40:60) was recovered unchanged under the same conditions, indicating that this ratio represents an equilibrium between the two isomers.

In vitro effects of clitocine on the growth of murine and human cancer cell lines

The clitocine β anomer and mixtures A and B were evaluated on the two murine and four human cancer cell lines by using the MTT assay (MTT = 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide).[12] All cells were sensitive to clitocine in a concentration range of 10^{-8}–10^{-6} M (20.5 nM ≤ IC_{50} ≤ 577.8 nM; Table 2). Each β/α ratio showed similar potent activities against all cell lines, with IC_{50} values in the sub-millimolar range. The highest potency was observed against L1210, with a continued decrease in susceptibility of the order: 3LL > K-562 > DU145 for mixture B. Except on K-562, a cell line not sensitive to doxorubicin, the cytotoxic agent used as a positive control, activities monitored for the β anomer and mixtures A and B were in the same order of magnitude (Table 2).

In vivo toxicity assay of mixture B in mice

To obtain mixture B in suitable quantity with sufficient solubility, a solution of hydroxypropyl-β-cyclodextrin (30% CD) was used; it is a vehicle commonly used in physiological tests. The in vitro activity of such a CD preparation of mixture B was found to be identical to those of other clitocine preparations on 3LL and L1210 cells (IC_{50} = 33.1 nM and 22.6 nM, respectively); the vehicle alone was inactive.

Table 2. Effects of the clitocine β anomer and mixtures A and B on the growth of murine and human cancer cell lines.

	IC_{50} [nM][a]					
	3LL	L1210	DU145	K-562	MCF7	U251
Clitocine	42.0 ± 7.0	35.6 ± 5.1	ND	ND	ND	ND
Mixture A	39.4 ± 5.8	20.5 ± 7.8	249.7 ± 12.6	342.4 ± 42.7	567.8 ± 3.5	442.1 ± 43.0
Mixture B	32.4 ± 0.3	27.0 ± 0.4	236.9 ± 13.9	184.7 ± 24.4	526.1 ± 139.4	428.6 ± 104.5
Doxorubicin[b]	75.8 ± 16.0	96.3 ± 29.3	173.4 ± 46.6	$> 8.8 \times 10^3$	305.4 ± 39.1	22.1 ± 1.8

[a] Values reflect the mean ± SD of at least three experiments. [b] Positive control. ND: not determined.

The toxic effect of treatment with mixture B was investigated in healthy mice. Injections of 25 and 75 mg kg^{-1} (ip) did not induce mortality after 14 days, but a dose of 150 mg kg^{-1} was sufficient for death in all mice (LD_{100}: 100% lethal dose). Daily injections of 50, 25, and 12.5 mg kg^{-1} resulted in complete mortality after 2, 5, and 11 days, respectively among members of the groups of 5–6 specimens tested. This was preceded by decrease in body weight (Figure 3). Succulent organs and the atrophy of adipose tissue and gall bladder were apparent in results of the highest doses throughout the histological study. Neither death nor loss of body weight were observed after 14 days of treatment with a dose of 6.25 mg kg^{-1} with the vehicle alone (control mice) (Figure 3).

In vivo effects of mixture B on tumor growth and survival time in 3LL- and L1210-tumor-bearing mice

As shown in Figure 4 for untreated 3LL-tumor-bearing mice, the first death was observed on day 9, and all animals were dead within 19 days. The daily dose of mixture B (0.5, 3, or 5 mg kg^{-1}) did not affect the lifespan (MST: mean survival time) of 12 days in comparison with the MST of control mice (Table 3). Mixture B did not significantly decrease tumor volume in 3LL-tumor-bearing mice after ip administration at any of the tested doses, as shown in Figure 5.

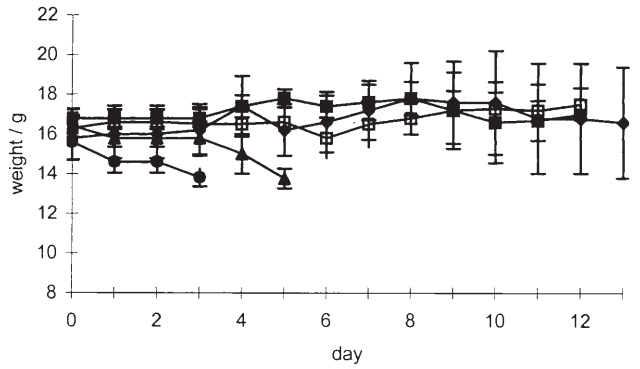

Figure 3. Effect of mixture B on body weight of healthy mice. The animals received daily ip injections of mixture B over 14 days at the indicated dose. Values are mean ±SD for: control, $n=6$ (□); 6.25 mg kg^{-1}, $n=5$ (◆); 12.5 mg kg^{-1}, $n=5$ (■); 25 mg kg^{-1}, $n=5$ (▲); 50 mg kg^{-1}, $n=5$ (●).

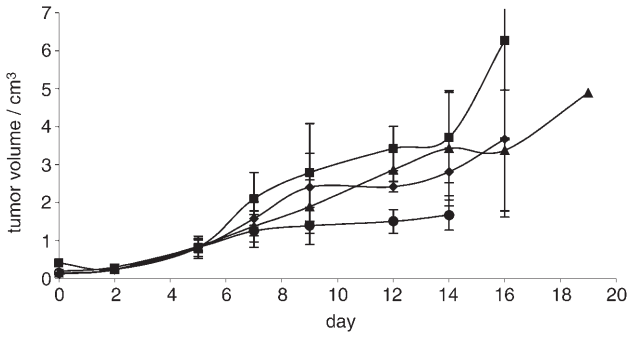

Figure 5. Effect of mixture B on tumor growth in 3LL-tumor-bearing mice. Mice were given daily ip injections of mixture B at the indicated dose. Values are mean ±SD for: control, $n=20$ (◆); 0.5 mg kg^{-1}, $n=7$ (■); 3 mg kg^{-1}, $n=21$ (▲); 5 mg kg^{-1}, $n=7$ (●). Statistic differences between groups were not observed, as determined by the Mann–Whitney test ($p>0.05$).

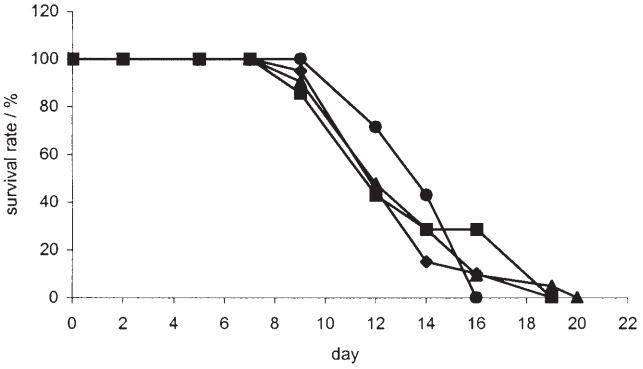

Figure 4. Effect of mixture B on the survival rate of 3LL-tumor-bearing mice. The animals received daily ip injections of mixture B over 14 days at the indicated dose. Data shown are for: control, $n=20$ (◆); 0.5 mg kg^{-1}, $n=7$ (■); 3 mg kg^{-1}, $n=21$ (▲); 5 mg kg^{-1}, $n=7$ (●).

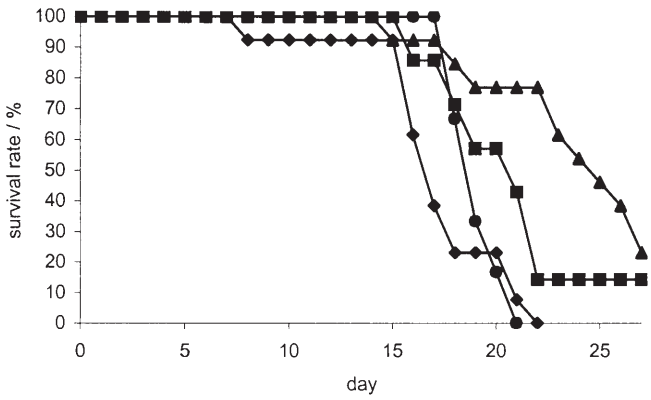

Figure 6. Effect of mixture B on the survival rate of L1210-tumor-bearing mice. Mice were given daily ip injections of mixture B at the indicated dose. Data shown are for: control, $n=13$ (◆); 0.5 mg kg^{-1}, $n=7$ (■); 3 mg kg^{-1}, $n=13$ (▲); 5 mg kg^{-1}, $n=6$ (●).

Table 3. Effects of mixture B on MST of 3LL- and L1210-tumor-bearing mice[a]

Mixture B [mg kg^{-1}]	MST (3LL) [days]	MST (L1210) [days]
Control[b]	12 ± 1 ($n=20$)	18 ± 1 [16; 19] ($n=13$)
0.5	12 ± 1 ($n=7$)	20 ± 1[c] ($n=7$)
3	13 ± 1 ($n=21$)	23 ± 1 [20; 26][c] ($n=13$)
5	12 ± 1 ($n=7$)	18 ± 0 ($n=6$)

[a] Values reflect the mean ±SD [confidence intervals] (n: number of animals). Data were analyzed with the log rank test. [b] Control mice were given a solution of 30% CD alone on the same schedule. [c] Differences were significant at $p<0.04$.

In contrast, the ip administration of mixture B (daily doses of 0.5 and 3 mg kg^{-1}) in L1210-tumor-bearing mice over a period of 28 days elicited a significant increase in the survival rate with respect to the control group (Figure 6). Surviving mice were present on day 28 in the groups given daily doses of mixture B at 0.5 and 3 mg kg^{-1}, whereas for groups given doses of 5 mg kg^{-1} and no mixture B, all mice were dead by day 21 and day 22, respectively. The mean survival times for groups treated with mixture B at 0.5 (MST = 20 days, $p<0.01$) and 3 mg kg^{-1} (MST = 23 days, $p<0.0004$) were statistically prolonged relative to control mice (MST = 18 days) (Table 3). As shown in Figure 7, significant variations ($p<0.01$) in body weight were observed with mixture B doses of 3 and 5 mg kg^{-1} between days 10 to 17 relative to the control mice, in which body weight increased daily. After macroscopic examination of euthanized 3LL- and L1210-tumor-bearing mice, no toxic effects of mixture B were detected, except the appearance of succulent intestines.

Apoptosis assays in 3LL and L1210 cells

To investigate the basis of the antiproliferative properties of clitocine in 3LL and L1210 cells, cell-cycle analyses were performed with flow cytometry. Neither mixture A nor B had an effect on the cell cycle of 3LL (data not shown). In contrast, an additional DNA peak before the G_0–G_1 peak (the "sub-G_1 peak", which may signal DNA fragmentation, a biological hallmark of apoptosis) was observed in L1210 cells treated with mixture A in a dose-dependent manner (Figure 8). A similar effect was

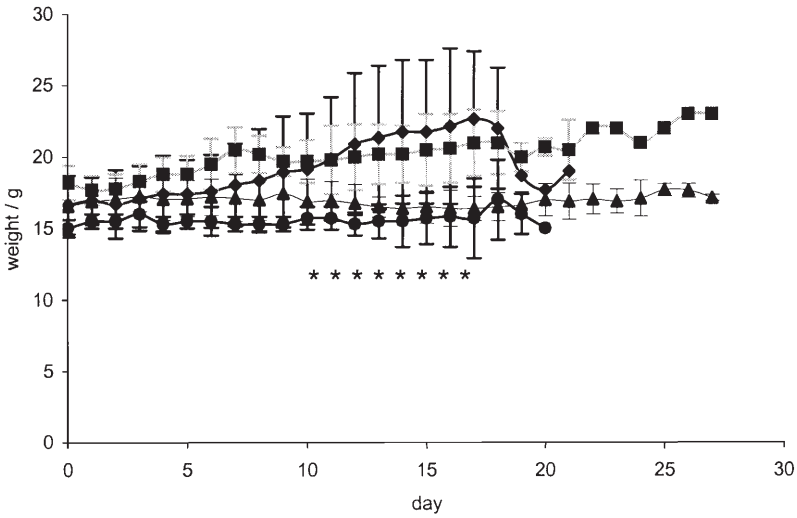

Figure 7. Effect of mixture B on body weight in L1210-tumor-bearing mice. Mice were given daily ip injections of mixture B at the indicated dose. Values are mean ±SD for: control, $n=13$ (◆); 0.5 mg kg^{-1}, $n=6$ (■); 3 mg kg^{-1}, $n=13$ (▲); 5 mg kg^{-1}, $n=7$ (●). Data were analyzed with the Mann–Whitney test. (*: Differences were significant at $p<0.01$.)

observed in L1210 cells treated with mixture B (data not shown).

Discussion

The search for new fungi-derived natural products with antitumor activity, particularly from Basidiomycetes, has become a matter of great significance.[2] A biologically guided fractionation from *L. inversa* led us to isolate clitocine as the major active compound. By using HPLC, the clitocine content was estimated to constitute ≈0.025% fresh mushroom weight. This exocyclic amino nucleoside, already isolated as the pure β anomer from *L. inversa*[7] possesses a structure similar to that of adenosine, as shown by the carbon resonances of the sugar moiety.

During this study, we isolated clitocine in various β/α anomer ratios (40:60 mixture A, or the β anomer) depending on the method of purification used (for example, the anomer conversion observed with chromatography on silica gel). The conversion of β-clitocine to the α anomer, which is thermodynamically more stable,[13] was found to be induced with acidic or aqueous conditions and heating. Thus, the deprotection of an isomeric mixture of ratio 2.8:1 with aqueous trifluoroacetic acid afforded an isomer ratio of 2:1.[14] The anomerization of ribose sugars could be promoted in acidic conditions.[15] Moreover, mixture B (β/α 80:20) was obtained after purification by column chromatography on silica gel. This support favored further anomerization, as the epimerization of 2,5-*cis*-substituted furanone to the respective *trans* isomer in silica gel has been reported.[16]

Clitocine showed cytotoxic activities with IC_{50} values in the sub-millimolar range on murine and human cancer cell lines. This pyrimidine compound exhibited greater potencies against 3LL and L1210 cells than doxorubicin, which was used as a positive control. IC_{50} values toward L1210 were in the same range as those reported for other human lymphoblastic leukemia cell lines (WI-L2, CCRF-CEM).[8] Accordingly, these results prompted us to evaluate clitocine on 3LL- and L1210-tumor-

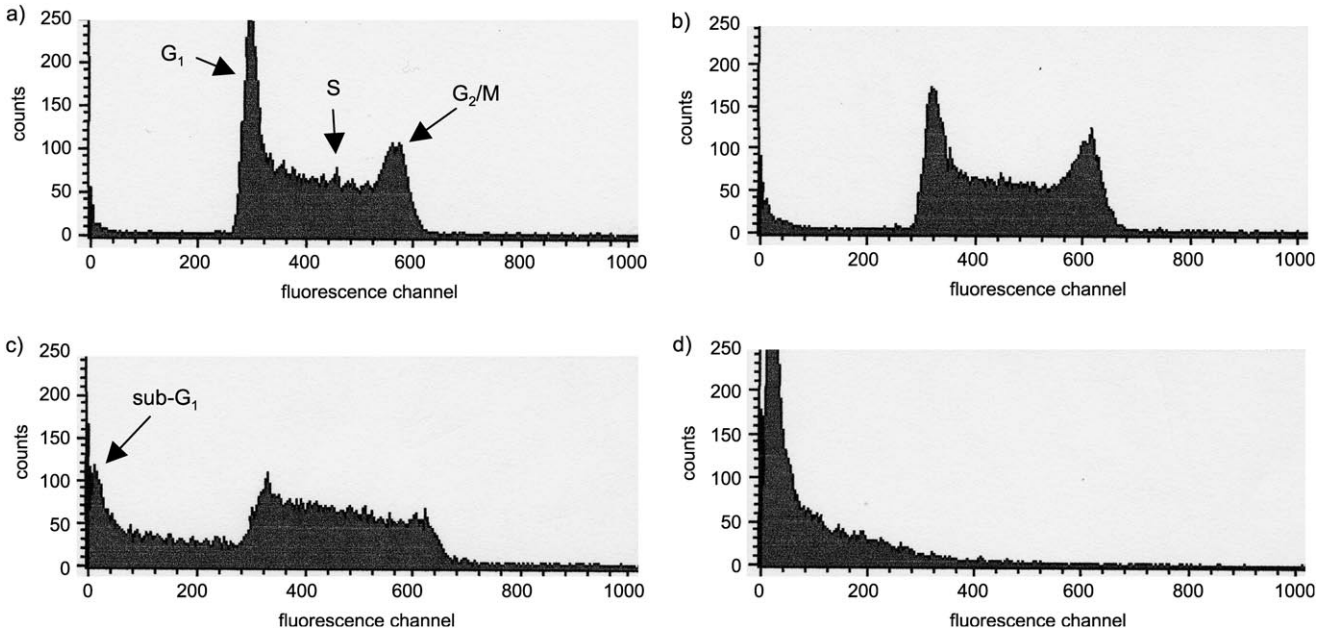

Figure 8. Flow-cytometric analyses of propidium iodide stained L1210 cells treated with mixture A; cells were cultured for 48 h in the absence or presence of mixture A at concentrations of: a) 0 (control), b) 13.5, c) 87, and d) 174 nM. Cell-cycle phases are indicated; sub-G_1 represents aneuploid cells and possibly cells with fragmented DNA.

bearing mice. The in vivo biological efficacy of clitocine was carried out with the compound synthesized in mixture B (β/α 80:20). The activity was assumed to be similar for both isomers, as in vitro activities appeared to be unmodified with respect to the isomer nature. Low aqueous solubility was overcome by using a solution of clitocine in 30% cyclodextrin. This allowed us to carry out the in vivo studies reported herein. Preliminary investigations permitted us to determine the nontoxic dose ($<6.25\ mg\,kg^{-1}$) of mixture B in the appropriate vehicle after daily ip injections in healthy mice over a period of 14 days. Mixture B injected at 0.5, 3, and 5 $mg\,kg^{-1}$ did not increase survival rate or lifespan of 3LL-tumor-bearing mice, but it did not cause any apparent adverse effects. Conversely, mixture B showed significant antitumor activity on L1210-tumor-bearing mice. An increased lifespan was observed at doses of 0.5 and 3 $mg\,kg^{-1}$, and a decrease in the development of ascites was observed at doses of 3 and 5 $mg\,kg^{-1}$.

Thus, the in vivo results confirm the higher in vitro sensitivity of L1210 cells toward clitocine in comparison with 3LL cell lines, as inhibitory compound concentrations were observed at 28 and 38 nM, respectively; this trend also applies to the methanolic extract of *L. inversa* (lifespan increase for L1210- and 3LL-tumor-bearing mice of 50 and 14%, respectively).[6] These findings are in agreement with those obtained from cell-cycle analysis by flow cytometry. Clitocine induced the appearance of a "sub-G_1 peak" (corresponding to cells with low DNA staining) in L1210 cells only. The antitumor activity of clitocine against L1210 cells may involve an apoptotic pathway. Potent cytostatic effects of clitocine against the leukemia WI-L2 cell line have previously been related to the inhibition of adenosine kinase, and this observation was explained by the structural similarity between clitocine and adenosine.[8] Future efforts will focus on the mode of action of this nucleoside.

In conclusion, clitocine was identified as the active substance from extracts of *L. inversa* and was demonstrated as one of the most potent cytotoxic compounds from Basidiomycetes.[2] However, this nucleoside exhibited a narrow therapeutic index, as an increase of mortality of L1210-tumor-bearing mice was observed at a dose of 5 $mg\,kg^{-1}$ and a loss of activity, at 0.5 $mg\,kg^{-1}$. These observations confirm the need to develop clitocine analogues as potential anticancer agents[17–19] to improve this index.

Experimental Section

General experimental procedures: General methods were previously described.[20] All chemical reagents were purchased from Aldrich (France). All solvents used were analytical grade. Culture reagents were from Biomedia (France). Hydroxypropyl-β-cyclodextrin (E0182) was obtained from Roquette (France). NMR spectra were recorded on a Bruker DMX spectrometer at 500 MHz (^1H) or 125 MHz (^{13}C) and on a Bruker AM 300 WB spectrometer at 300 MHz (^1H) or 75 MHz (^{13}C). HPLC experiments were conducted on a Hypersil BDS C-18 column (250×10 mm, 5 μm) with a Constametric 4100 pump (Thermo Separation Products, France) and a diode array detector (DAD 440, Kontron Instruments). Column chromatography was performed with silica gel (63–200 μm, Kieselgel 60, Merck) and C-18 Chromabond® column (Macherey–Nagel, cat. no. 730602). TLC plates were eluted with various solvent systems (expressed in volume percents) and observed under UV light ($\lambda = 254$ and 365 nm) after heating with sulfuric anisaldehyde, iodine, or ammonium metavanadate.

Mushroom material: *L. inversa* fruiting bodies were collected in autumn 1998 in a mixed forest near Rennes, France. Fresh mushrooms were cleaned, sliced, frozen, and kept at −20 °C until extraction. A voucher specimen is kept in our laboratory for future reference.

Isolation of clitocine from the methanolic extract of *L. inversa* fruiting bodies: Frozen fruiting bodies of *L. inversa* (5 kg) were extracted with 3×10 L methanol (Carbo Erba, cat. no. 4148161). Evaporation under decreased pressure afforded 152 g of a crude extract (I). Two methods were used to isolate clitocine from this extract, as follows:

First method (Figure 1): A fraction of extract I (76 g in 1 L H_2O) was successively extracted with petroleum benzene (3×500 mL) and methyl ethyl ketone (3×500 mL) to afford petroleum benzene soluble extract (PB, 2.3 g), methyl ethyl ketone soluble extract (MEK, 10.2 g), and residual extract (RE, 64 g) after evaporation under decreased pressure. The MEK extract was chromatographically separated on a silica gel column (120×75 mm, CIL Cluzeau, 35–70 μm) by vacuum liquid chromatography using gradient elution with CH_2Cl_2, EtOAc, and MeOH, leading to fractions A, B, C, and D. Fraction D (3.2 g) was separated on a silica gel column (75×107 mm) and eluted with CH_2Cl_2/MeOH 80:20 to afford eight fractions (E–L, 20 mL each). Fraction J (225 mg) was purified on a silica plate by using CCC (Chromatotron 8924, Harrison Research) with silica gel (2 mm thick PF_{254} with gypsum, Merck) and a flow rate of 7 $mL\,min^{-1}$. The solvent gradient of CH_2Cl_2/MeOH (from 90:10 to 0:100) under these conditions afforded 12 fractions (O–Z, 15 mL each). The 10% methanolic eluate (fraction S, 30.6 mg) was purified with HPLC by using a Hypersil BDS C-18 column (250×10 mm, 5 μm, 500 μL injection loop) with MeOH/H_2O (20:80) at a flow rate of 1.5 $mL\,min^{-1}$. Clitocine (11.5 mg) was obtained in a 40:60 mixture of β/α anomers (mixture A).

Second method: The second portion of the crude extract I (76 g) was lyophilized and kept under N_2. An aliquot (20 g) was dissolved in distilled water (150 mL). This aqueous fraction was successively extracted with *n*-hexane (3×150 mL), chloroform, then EtOAc. Each organic phase was dried with anhydrous Na_2SO_4 and concentrated under decreased pressure. The ethyl acetate soluble extract (508 mg) was separated by using DCC (Büchi 670) with $CHCl_3$/MeOH/H_2O 13:7:4 as the mobile phase (1 $mL\,min^{-1}$) and EtOAc as stationary phase in a descending way. Ten fractions were collected, and fraction I (37 mg) was separated on a C-18 column (7×65 mm) with MeOH/H_2O 5:95 to yield clitocine (100% β anomer, 23 mg).

Clitocine (6-amino-5-nitro-4-(β-D-ribofuranosylamino)pyrimidine): Clitocine was obtained as an amorphous pale yellow powder. $R_f = 0.55$ (CH_2Cl_2/MeOH 80:20). It turned dark green with the sulfuric anisaldehyde reagent, dark brown with iodine, and yellow with ammonium metavanadate. HPLC (C-18, MeOH/H_2O 20:80, 0.5 $mL\,min^{-1}$) $t_R = 7.4$ min; mp: 230–232 °C. UV/Vis (methanol): λ_{max} [ε] = 212 [22962], 230 (shoulder), 332 nm [8611]; IR (KBr): $\tilde{\nu} = 3438$ (N–H), 3332 (O–H), 1650 (C=C, C=N), 1520 (NO_2), 1276 cm^{-1} (C–O). HRESI MS (*m/z*) calcd for $C_9H_{13}N_5O_6$ [*M*+Na]$^+$: 310.0763, found: 310.0766. The ratio of each anomer was determined by ^1H NMR spectroscopy based on integration of the anomeric proton signals.

Clitocine was obtained as the pure β anomer by using a DCC system. This compound exhibited spectral data (IR, ^{1}H and ^{13}C NMR) similar to published values for clitocine.[7]

Mixture A was obtained as a 40:60 mixture of β/α anomers from fraction S by using HPLC. ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 3.47$ (AB, 2H, 5′-H), 3.81 (m, 1H, 4′-H), 3.93 (m, 1H, 2′-H), 4.07 (m, 1H, 3′-H), 4.74 (t, $J = 5.4$ Hz, 0.6H, 5′-α-OH), 4.98 (br s, 0.4H, 3′-β-OH), 5.09 (t, $J = 4.3$ Hz, 0.4H, 5′-β-OH), 5.24 (br s, 1H, 2′-OH), 5.63 (br s, 0.6H, 3′-α-OH), 5.79 (dd, $J = 7.8$ and 3.4 Hz, 0.4H, 1′-β-H), 6.03 (dd, $J = 8.5$ and 4.8 Hz, 0.6H, 1′-α-H), 8.00 (s, 1H, 2-H), 8.57 (s, 2H, 6-NH$_2$), 9.29 (d, $J = 7.8$ Hz, 0.4H, 4-β-NH), 9.96 ppm (d, $J = 8.5$ Hz, 0.6H, 4-α-NH); ^{13}C NMR (125 MHz, [D$_6$]DMSO): β and α anomers 40:60, $\delta = 60.3$ (C5′β), 61.5 (C5′α), 69.9 (C3′β), 70.0 (C3′α), 71.1 (C2′α), 74.9 (C2′β), 80.4 (C4′α), 83.0 (C1′α), 83.9 (C1′β), 86.1 (C4′β), 111.8 (C5β), 112.1 (C5α), 155.8 (C4β), 156.5 (C4α), 158.6 (C6β), 158.7 (C6α), 159.3 (C2β), 159.4 ppm (C2α).

Synthesis of mixture B: Mixture B was synthesized according previously described procedures.[8] An amorphous pale-yellow powder (320 mg, 7% yield) was obtained as a 80:20 mixture of β/α isomers after column chromatography on silica gel by using CH_2Cl_2/acetone 95:5 instead of a fractional recrystallization from methanol, which was described to afford the pure β anomer.

Study of the anomerization of clitocine: An aliquot (5 mg) of β anomer and mixtures A and B was adsorbed on silica with methanol or CH_2Cl_2/MeOH 80:20 for 30 min or 24 h, or dissolved in water at 37 °C for 48 h. The ratio of each anomer was determined by ^{1}H NMR ([D$_6$]DMSO) and comparison of the integration of anomeric proton signals.

Dosage of clitocine by HPLC: Mixture B was used as a standard to determine the clitocine content in the crude extract I, the MEK extract, and fraction D. A stock solution of clitocine in MeOH/H_2O 90:10 (1 mg mL^{-1}) was used, along with solutions of calibration standard (10–1000 μg mL^{-1} clitocine). Solutions from extract I, the MEK extract, and fraction D were prepared at 2.30 mg mL^{-1}. Standard or extract solutions (loading volumes of 20 μL each) were subject to HPLC with a Hypersil BDS C-18 column (250×4.6 mm, 5 μm) and eluted with the mobile phase MeOH/H_2O 40:60 at a flow rate of 0.5 mL min^{-1}. The eluates were monitored in the range $\lambda = 190$–400 nm.

Cell culture and biological studies: Two murine cancer cell lines: L1210 (lymphocytic leukemia, ATCC CCL-219) and 3LL (murine Lewis lung carcinoma, CRL-1642), and four human cancer cell lines: K-562 (chronic myelogenous leukemia, ATCC CCL-243), DU145 (brain metastasis of a prostate carcinoma, ATCC HTB-81), MCF7 (breast adenocarcinoma, ATCC HTB-22), and U251 (glioblastoma, RCB-0641) were used. The cells were maintained as previously described.[6] For the adherent 3LL, DU145, U251, and MCF7 lines, cells were seeded the day before any treatment.

Mother liquors of the crude methanolic extract (5 mg mL^{-1}) of fractions A–Z or of pure β anomer and mixtures A and B were prepared in DMSO and used for all in vitro experiments.

Cytotoxicity: Cytotoxic activity was determined in murine (3LL and L1210) and human (K-562, DU145, MCF7, and U251) cancer cell lines with an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay as described previously.[6]

Flow cytometry: 3LL and L1210 cells were seeded at 1×10^5 and 2.5×10^5 cells mL^{-1}, respectively, in 6-well dishes in RPMI 1640 medium containing fetal calf serum (10%), L-glutamine (2 mM), penicillin (50 UI mL^{-1}), and streptomycin (50 μg mL^{-1}) in the absence or presence of mixtures A or B (added one day after seeding for 3LL). After treatment for 48 h, they were harvested, washed in phosphate-buffered saline (PBS), then fixed with ice-cold ethanol (70%) overnight. After washing in PBS, the cells were treated with RNase (50 μg mL^{-1} in PBS) at 37 °C for 30 min, then stained with propidium iodide (50 μg mL^{-1}). Cells were analyzed for their DNA content by flow cytometry with a Coulter Epics Elite (Coultronics, USA) as previously described.[21]

Animals: Female C57Bl/6 and DBA/2 mice (6 weeks old) with an average weight of 20 g were obtained from Elevage Janvier (France) and handled as previously described.[6] All animal experiments were conducted under the institutional guidelines and according to the law.

Acute toxicity study: A stock solution of mixture B (5 mg mL^{-1}) and further dilutions were prepared in 30% CD with 0.9% NaCl. Four groups of healthy mice ($n = 6$ per group) received ip injections of mixture B at three doses (25, 75, or 150 mg kg^{-1}) or of the vehicle alone. Toxic symptoms and mortality were recorded hourly during the period of 8 h following injection, and 24 h after injection.

Tolerance study: Over a course of 14 days, five groups of healthy mice ($n = 5$ per group) received daily ip injections of mixture B at four doses (6.25, 12.5, 25, or 50 mg kg^{-1}) or of the vehicle alone. Behavioral responses, mortality, and weight were recorded daily. Food and water consumption was determined every week. Mice were sacrificed by decapitation, and macroscopic analysis of the organs (lung, heart, intestines, genital tract, gall bladder, liver) was carried out.

Antitumor effects in 3LL- and L1210-tumor-bearing mice: In a first set of experiments, mice were inoculated with 7×10^5 3LL cells in the right hind leg for solid tumor development. Once a palpable tumor was established, (≈5 days after graft), the animals were separated into random groups for treatment. Each group received a daily injection of mixture B at 0.5 mg kg^{-1} ($n = 7$), 3 mg kg^{-1} ($n = 21$), 5 mg kg^{-1} ($n = 7$), or of the vehicle alone ($n = 20$). In a second set of experiments, mice were inoculated ip with 2×10^5 L1210 cells. Two days after tumor-cell inoculation, the animals were treated as described above. The number of animals per group was as follows: control mice ($n = 13$), mixture B 0.5 mg kg^{-1} ($n = 7$), 3 mg kg^{-1} ($n = 13$), and 5 mg kg^{-1} ($n = 6$). Tumor growth was recorded over 28 days for 3LL-tumor-bearing mice, and for L1210-tumor-bearing mice as previously described.[6]

Statistical analyses: Data are given as mean values ± SD of three experiments. Survival data were calculated with the Kaplan–Meier method, and statistical significance was assessed by the log rank test. Comparison of body weight and the tumor growth of tumor-bearing mice was performed within the same give group by using the Wilcoxon nonparametric test, and between different groups with the Mann–Whitney test. Survival analyses were done with the SPSS software (SPSS, Chicago, USA).

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