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DNA-PROTECTOR AND CYTOTOXIC ACTIVITY OF SECOISOLARICIRESINOL DIGLUCOSIDE DERIVATIVES

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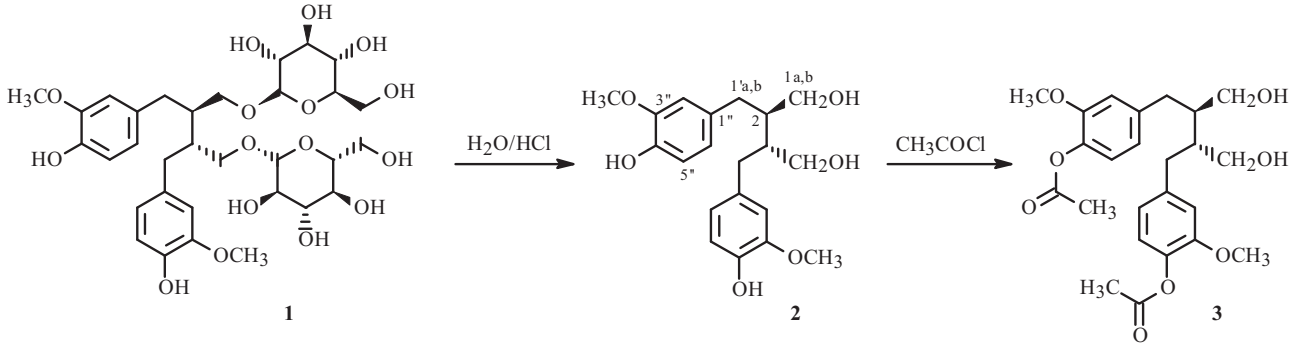
The relationship between the chemical structure and DNA-protector and cytotoxic activities of phenylpropane lignans was studied. Analogs were synthesized from the lignan secoisolariciresinol diglucoside (SDG) isolated from Linum usitatissimum *seeds. It was shown that SDG derivatives secoisolariciresinol and secoisolariciresinol-4′,4″-diacetate, which were not glucosides, exhibited greater cytotoxic activity* in vitro *than the starting lignan. The cytotoxicity was slightly elevated upon acetylation of the aglycon phenol hydroxyls whereas the DNA-protector activity decreased substantially. The DNA-protector activity of the derivative without the carbohydrate was slightly greater than that of the starting SDG. This was explained by binding of free radicals by the butanediol hydroxyls. It was proposed that the activity of the cytotoxic derivatives was mediated by induction of apoptosis of the tumor cells.*

Keywords: lignans, secoisolariciresinol diglucoside, derivatives, benzidine, DNA-protector activity, tumor cells, apoptosis, cytotoxicity.

Lignans are natural biologically active compounds that consist of two phenylpropane units joined by a *β–β′*-bond. Many representatives of this class exhibit antioxidant and antitumor properties [1]. Natural lignans are also interesting because of their ability to be used as templates for designing drugs with various biological activities (antitumor, antiviral, antifungal) [2]. Clear examples of medicines based on natural lignan are podophyllotoxin and its derivatives, the antitumor drugs etoposide and teniposide [2].

Herein we report results from a study of the relationship between the chemical structure and biological activity of phenylpropane lignans that were synthesized from secoisolariciresinol diglucoside (SDG) isolated from seeds of *Linum isutatissimum*. We investigated the relationship of the DNA-protector and cytotoxic activity of the natural lignan SDG (**1**) and its derivatives to the chemical structure.



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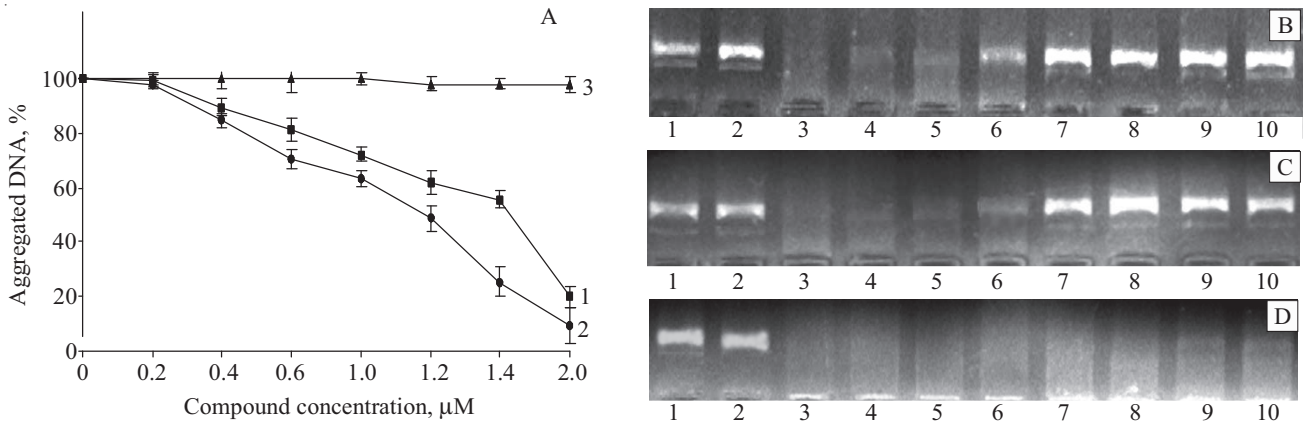

Fig. 1. DNA-protector activity of lignans for products of benzidine (diimine) peroxidase oxidation: A: plot of amount of aggregated Phasmid DNA as a function of lignan concentration (SDG, 1; secoisolariciresinol, 2; secoisolariciresinol-4′,4″-diacetate, 3); B, C, D: electrophoregrams of treated pDNA (38,000 bp length) in agarose gel (0.9%) [7]: buffer + DNA (0.0368 μg/μL) (1); buffer + DNA (0.0368 μg/μL) + horseradish peroxidase (0.02 μM) + H_2O_2 (0.2 μM) (2); DNA (0.0368 μg/μL) + horseradish peroxidase (0.02 μM) + benzidine (5 μM) + H_2O_2 (0.2 μM) (3); components of 3 + SDG (B), secoisolariciresinol (C), and secoisolariciresinol-4′,4″-diacetate (D) at concentrations 0.2, 0.4, 0.6, 1.0, 1.2, 1.4, and 2.0 μM, respectively (4-10).

We have previously reported in detail the procedure for isolating the starting compound from plant material [3]. Herein we report the synthesis of secoisolariciresinol (**2**) from SDG via its acid hydrolysis. This compound was subsequently transformed into the diacetyl derivative by acetylation of the phenol hydroxyls to give secoisolariciresinol-4′,4″-diacetate (**3**). The structures of the products were confirmed using IR and NMR spectroscopy and mass spectrometry.

The DNA-protector activity of the lignans was assessed using a model for DNA damage by peroxidase oxidation products of benzidine, which is an indirect action carcinogen [4–6]. Benzidine in this model was converted by horseradish peroxidase in the presence of H_2O_2 into a primary cation–radical, further oxidation of which produced a monoprotonated diimine [5]. The oxidation product reacted with DNA purines and eventually formed cross links in the biomolecule. However, this decreased its electrophoretic mobility (Fig. 1B, 1C, 1D, track 3). However, the aggregation of DNA was gradually inhibited if SDG and secoisolariciresinol were added beforehand at increasing concentrations to the reaction mixture (Fig. 1B, 1C, tracks 4–10).

The mechanism of action of the lignans apparently involved competition among them for radicals produced by benzidine oxidation. This prevented DNA macromolecules from aggregating and preserved their electrophoretic mobility. The DNA-protector activity of the lignans in this instance was probably due to their antiradical properties. A plot of the DNA emission intensity in the electrophoregrams revealed that the percent of aggregated DNA depended on the concentration of the corresponding lignan (Fig. 1A).

The lignan concentrations that prevented DNA aggregation by 50% (IC_{50}) were determined from the plot in order to compare their antiradical activities.

Figure 1 shows that natural lignan (IC_{50} = 1.54 ± 0.1 μM) and secoisolariciresinol (IC_{50} = 1.17 ± 0.1 μM) exhibited antiradical activity in this conceentration range. This was ascribed to the presence of phenol hydroxyls in their structures. Secoisolariciresinol-4′,4″-diacetate did not exhibit antiradical activity in this concentration range (Fig. 1D). This was probably due to acetylation of the phenol hydroxyls. Antioxidant activity disappeared analogously for acetylation products of the known antioxidant quercetin [8]. Phenol hydroxyls are known to act as donors of labile H atoms. Therefore, they react readily with free radicals formed during the course of the reaction [9]. The phenoxyl radical, the initial oxidation product of phenol, has characteristic resonance stabilization. This hinders its further reaction with other reagents and inhibits a chain reaction. The antiradical activity of the derivative without the carbohydrate part (secoisolariciresinol) was slightly greater than that of starting SDG. This was possibly due to an additional contribution from the aliphatic alcohol hydroxyls of the butanediol group to free-radical binding [10]. Despite the lower stability of the lignan alkoxy radicals compared with the phenoxy radicals, it is known that they are rather stable and do not exhibit oxidant activity [10]. It was reported that the antioxidant activity of quercetin 3-β-D-glucoside was lower than that of the quercetin aglycon [11].

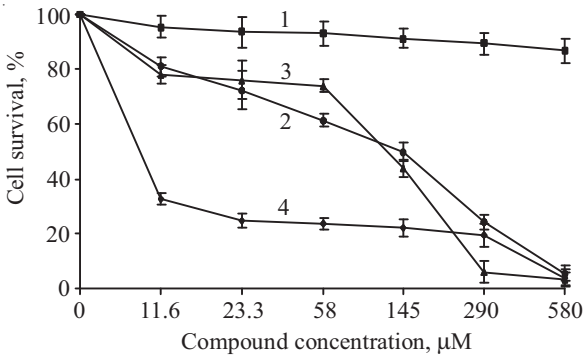

Fig. 2. Cytotoxic activity of SDG and its derivatives for Raji tumor cells: SDG (1), secoisolariciresinol (2), secoisolariciresinol-4′,4″-diacetate (3), etoposide (4).

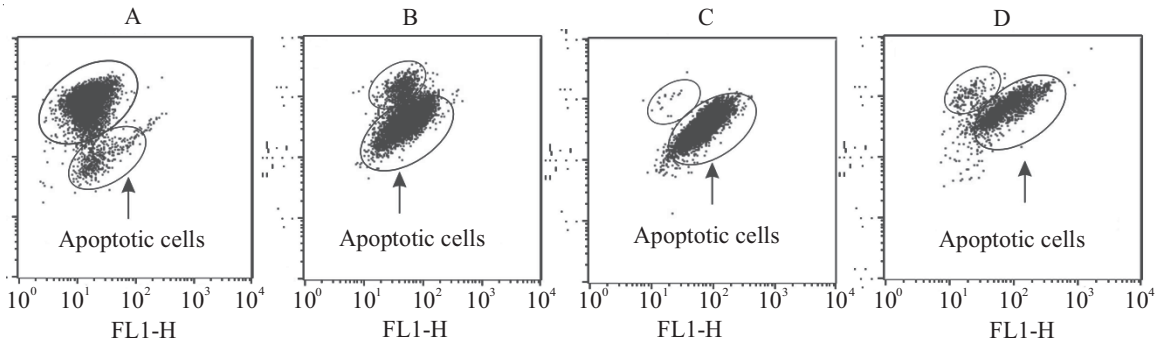

Fig. 3. Cytograms of Raji cell distribution using CMXRos fluorescence probe: control cells (A), cells after 24 h incubation with 580 μM of secoisolariciresinol, secoisolariciresinol-4′,4″-diacetate, and etoposide, respectively (B, C, D).

The cytotoxic activity of natural SDG and its derivatives was determined against the Raji B-lymphoblastoid tumor cell line (ACC 319, DSMZ-collection) using the MTT-test [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] [12]. The antitumor drug etoposide was used as the standard cytotoxic agent. Cells containing lignans and etoposide at concentrations 11.6, 23.3, 58, 145, 290, and 580 μM were incubated and then treated with MTT. The number of surviving cells was determined spectrophotometrically. Mitochondrial dehydrogenase in leukemia cells surviving incubation with the test compounds metabolized MTT to form formazan. The optical density of the formazan solution was directly proportional to the amount of surviving cells that caused the reaction. The data were plotted to produce a graph of the percent leukemia cells surviving vs. the concentration of the corresponding compound (Fig. 2). The lignan concentrations leading to the death of 50% of the cells (IC_{50}) were determined from the graphs in order to compare the cytotoxic activities of the lignans and the reference drug.

We found that SDG did not exhibit cytotoxic activity *in vitro* against the Raji cell line at concentrations less than 580 μM. Figure 2 shows that SDG derivatives without the glucoside part in their structures exhhibited significant cytotoxic activity *in vitro* that increased slightly depending on acetylation of the aglycon phenol hydroxyls because the activity of secoisolariciresinol-4′,4″-diacetate (IC_{50} = 135 ± 2.4 μM) was not significantly different from that of secoisolariciresinol (IC_{50} = 143 ± 3.5 μM). The most potent cytostatic was etoposide (IC_{50} < 11.5 μM).

Flow cytometry was also used to estimate the cytotoxic activity from apoptosis induction by the SDG derivatives and etoposide against the same cell line. The number of apoptotic cells was recorded by scanning with a laser at 448 nm after incubation with the compounds at concentrations 58, 145, 290, and 580 μM for 24 and 48 h. The level of apoptosis induction was determined from the decrease of mitochondrial transmembrane potential (detected by fluorescence from CMXros probe) (Fig. 3).

It was found that secoisolariciresinol and secoisolariciresinol-4′,4″-diacetate induced apoptosis in this concentration range. Table 1 presents the results.

TABLE 1. Level of Raji Tumor Cell Apoptosis Induction by SDG Derivatives and Etoposide

Compound	IC_{50}, μM	
	24 h	48 h
Secoisolariciresinol	415.93 ± 5.2	294.45 ± 3.4
Secoisolariciresinol-4′,4″-diacetate	382.4 ± 4.1	227.52 ± 2.8
Etoposide	409.6 ± 3.8	93.3 ± 5.5

Table 1 shows that the level of apoptosis induction was higher for secoisolariciresinol-4′,4″-diacetate than for secoisolariciresinol. Apoptosis induction was strongest for etoposide after incubation for 48 h.

The manifestation of cytotoxic activity due to removal of the glucoside part in the SDG derivatives could be explained by the reduced hydrophilicity, which facilitated greater penetration through the cell membrane. An analogous result was obtained in research with the known flavonoid antioxidant quercetin. The quercetin aglycon exhibited significant antiproliferative activity [13] whereas its 3-β-D-glucoside did not at concentrations less than 463.4 μM [14]. Both compounds exhibited high antioxidant activity [14].

It was demonstrated previously that the lignan secoisolariciresinol exhibited antiproliferative activity against HT-1080 fibrosarcoma cells [15], KB-16, A-549, and HT-29 tumor cell lines [16], and the Caco-2 colon adenocarcinoma cell line [17].

The slight increase in cytotoxic activity of secoisolariciresinol-4′,4″-diacetate as compared with secoisolariciresinol could also probably be explained by increased lipophilicity due to acetylation of the phenol hydroxyls. Analogous results were obtained earlier [18]. Acetylation of the phenol hydroxyls in epigallocatechin-3-gallate produced acylated derivatives that inhibited much more effectively proliferation of tumor cells, including leukemia, and induced more strongly apoptosis [18].

Thus, secoisolariciresinol and secoisolariciresinol-4′,4″-diacetate without the glucoside part of the structure exhibited significant cytotoxic activity *in vitro* as compared with the starting lignan. The cytotoxic activity increased slightly depending on acetylation of the aglycon phenol hydroxyls whereas the DNA-protector activity decreased somewhat. The DNA-protector activity of the derivative without the carbohydrate part was slightly greater than that of starting SDG. This was probably explained by additional involvement of the butanediol hydroxyls in free-radical binding. The activity of the cytotoxic derivatives was probably mediated by induction of apoptosis of the tumor cells. Our results indicate that secoisolariciresinol lignans may be interesting for prevention of carcinogenesis and treatment of oncological diseases.

EXPERIMENTAL

NMR spectra were recorded on a Bruker Avance instrument. IR spectra were taken on a Nexus spectrometer (Thermo Nicolet). SDG (**1**) was isolated and identified by the literature method [3]. The purity and identity of the products were monitored by TLC and HPLC. The analytical conditions and eluent compositions were the same as before [3].

Secoisolariciresinol (2). SDG lignan (0.1005 g, 0.15 mmol) was dissolved in water (8 mL) and treated with HCl solution (4 mL, 6 M). The hydrolysis was carried out for 4 h on a water bath at 98°C. The mixture was left to cool at room temperature for 24 h. Compound **2** precipitated as a light pink solid that was filtered off and washed with cold water. Yield 0.027 g (51%), mp 109.7–110.2°C (lit. [19] mp 109–110°C). Mass spectrum (ESI, m/z, I_{rel}, %, positive-ion mode): $[M + H]^+$ 363.82 (31.3).

IR spectrum (KBr, ν, cm^{-1}): 3350 (OH), 2939, 2911, 2847 (CH_3–O–Ar), 1605, 1518, 1464, 1433, 1378 (C_{Ar}–O), 1267, 1155, 1120, 1069 (C–O–C), 1038, 879 (Ar), 822 (Ar), 801, 623, 564.

PMR spectrum (400.13 MHz, $CDCl_3$, δ, ppm, J/Hz): 2.17 (2H, m, H-2), 2.51 (2H, dd, J = 13.7, 7.9, H-1′a), 2.57 (2H, dd, J = 13.7, 6.4, H-1′b), 3.53 (2H, dd, J = 8.6, 5.4, H-1a), 3.80 (6H, s, OCH_3), 3.92 (2H, dd, J = 8.6, 6.5, H-1b), 5.73 (2H, br.s, Ar–OH), 6.49 (2H, d, J = 1.8, H-2″), 6.57 (2H, dd, J = 7.9, 1.8, H-6″), 6.79 (2H, d, J = 7.9, H-5″).

^{13}C NMR spectrum (100.613 MHz, $CDCl_3$, δ, ppm): 39.1 (C-1′), 46.4 (C-2), 55.8 (OCH_3), 73.2 (C-1), 111.1 (C-2″), 114.2 (C-5″), 121.3 (C-6″), 132.6 (C-1″), 143.9 (C-4″), 146.5 (C-3″).

Secoisolariciresinol-4′,4″-diacetate (3). Compound **2** (0.054 g, 0.15 mmol) was treated with acetylchloride (0.9 mL, 12.7 mmol). The reaction was carried out with stirring at room temperature for 12 h. The mixture was evaporated at reduced pressure and separated using preparative chromatography on Silica Gel G plates (Analtech UniplateTM, USA). The eluent was EtOAc:petroleum ether (2:3). Yield 0.042 g (63%). Mass spectrum (ESI, *m*/*z*, I_{rel}, %, positive-ion mode): $[M + H]^+$ 447.72 (100).

IR spectrum (KBr, ν, cm^{-1}): 3437 (OH), 2935, 2851 (CH_3–O–Ar), 1764 (C=O), 1605, 1511, 1466, 1420, 1370 (C_{Ar}–O), 1270, 1199, 1151, 1122, 1033 (C–O–C), 903 (Ar), 825 (Ar), 791, 737, 646, 602, 514, 465.

PMR spectrum (400.13 MHz, $CDCl_3$, δ, ppm, J/Hz): 2.21 (2H, m, H-2), 2.30 (6H, s, $COCH_3$), 2.58 (2H, dd, J = 13.8, 8.2, H-1′a), 2.68 (2H, dd, J = 13.8, 6.1, H-1′b), 3.54 (2H, dd, J = 8.6, 5.6, H-1a), 3.78 (6H, s, OCH_3), 3.93 (2H, dd, J = 8.6, 6.5, H-1b), 6.62 (2H, dd, J = 8.4, 1.8, H-6″), 6.67 (2H, d, J = 1.8, H-2″), 6.92 (2H, d, J = 8.4, H-5″).

Determination of DNA-protector Activity. We used Phasmid DNA containing a cloned fragment of human chromosomal DNA (38,000 bp) (obtained from Phasmid clone WI2-1799L7, which is part of human Phasmid library of clones WIBR-2; source, Children's Hospital Oakland Research Institute, BACPAC Resources Center, USA). pDNA was isolated from a recombinant strain of *E. coli* using a QIAprep Spin Miniprep Kit (QIAGEN Ltd., Great Britain) according to the manufacturer's instructions. The concentration was measured with a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA) at 260 nm. The DNA in Tris-EDTA buffer was diluted beforehand several times with water. The stock solution of Tris-EDTA buffer had concentration 0.184 μg/μL and was stored at –20°C until used.

Next, an Eppendorff tube was charged successively with Tris-HCl buffer (4 μL, 0.1 M, pH 7.4) containing Tween 20 (0.05%), DNA (0.184 μg/μL), horseradish peroxidase (0.1 μM), benzidine (25 μM), and H_2O_2 (1 mM). The mixture volume was 20 μL. The mixture was incubated for 60 min at 25°C, treated with bromphenol blue solution (6×, 4 μL), placed (12 μL) on a track in agarose gel (0.9%) prepared with TAE-buffer (40 mM Tris-acetate and 1 mM EDTA, pH 7.4) containing ethidium bromide (0.5 μg/mL). Electrophoresis was carried out at 60 V for 1 h. The electrophoretic mobility of the DNA was analyzed using a trans-illuminator. The electrophoregrams were photographed. The emission intensity of the DNA bands was estimated using the TotalLab V2.0 program (Nonlinear Dynamics Ltd., Great Britain).

Determination of Cytotoxic Activity. The sensitivity of leukemia cells to the test compounds was determined using the MTT-test according to the literature method [12]. Cells were resuspended to a concentration of 2×10^5 cells/mL in RPMI-1640 complete medium containing fetal calf serum (10%), penicillin (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM). Cell suspension in medium (80 μL) was distributed evenly into a 96-well plate among wells containing different dilutions of test compounds in 20 μL with each sample doubled, a row of wells with cell suspension (survival control), and wells containing only RPMI complete medium (medium control). The SDG solution (2.9 mM) was prepared in distilled water. Stock solutions (29 mM) of secoisolariciresinol and secoisolariciresinol-4′,4″-diacetate were prepared in EtOH (96%). Etoposide solution (33.9 μM) was prepared in RPMI-1640 medium. Then compounds were diluted with physiological solution to concentrations of 2.9, 1.45, 0.725, 0.29, 0.116, and 0.058 mM. The final concentrations of the compounds in the wells were 11.6, 23.3, 58, 145, 290, and 580 μM, respectively. The EtOH concentration in the wells was less than 2%. Furthermore, a cell-survival control in medium containing EtOH (2%) was set up. Plates were cultivated in a humid incubator with 5% CO_2 for 3 d at 37°C. Then, wells were treated with MTT (5 μg/mL of RPMI-1640 medium) and held for 4 h under the same conditions.

The formazan crystals that formed after incubation with MTT were dissolved in HCl solution (100 μL, 2 N) in isopropanol. The contents of each well were thoroughly mixed. The optical density of the solutions was determined at 540 nm in the plate on a spectrophotometer (ELISA-reader, Sanofi Diagnostic Pastuer PR 2100, France). Cell survival (CS) was determined using the formula:

$$CS(\%) = (ODw - ODc)/(ODs - ODc) \times 100\%,$$

where ODw is the optical density of test wells; ODs, of survival-control wells; ODc, control wells without added cells and EtOH.

The ODw value was the arithmetic average of two determinations for each dilution. Results were considered adequate if ODs – ODc > 0.05.

Determination of Cytotoxic Activity Using Apoptosis Induction and Flow Cytometry. Reaction mixtures (1000 μL) containing a suspension of Raji cell line (5×10^5 cells/mL) in RPMI-1640 complete medium and test compounds

(secoisolariciresinol, secoisolariciresinol-4′,4″-diacetate, etoposide) at concentrations of 58, 145, 290, and 580 μM were placed into a 24-well plate and cultivated for 24 and 48 h at 37°C in a humid incubator containing 5% CO_2. Cells were washed immediately before analysis with PBS phosphate buffer, incubated at 37°C for 30 min with added CMXRos fluorescence probe (Molecular Probes, USA) at a concentration of 25 nM, and washed again with buffer. Measurements were made in a FACScan flow cytometer (Becton Dickinson, USA). An Ar laser (488 nm, 15 mW) was used to excite fluorescence. Each analyzed sample had at least 10,000 cells.

Data Processing. Results from at least three independent experiments are given as the average ± the error of the mean. The program Excel 2003 (Microsoft Office) was used to construct experimental graphs and perform the mathematical processing.

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