Cytotoxic Effect of Laxaphycins A and B on Human Lymphoblastic Cells (CCRF-CEM) Using Digitised Videomicrofluorometry

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Abstract. Laxaphycin A (laxa A) and Laxaphycin B (laxa B), cyclic peptides isolated from the terrestrial blue-green alga Anabaena laxa or the marine cyanobacterium Lyngbya majuscula have antifungal and cytotoxic activities. We used numerical videomicrofluorometry and a protocol of multiple labelling with Hoescht 33342 (nuclear DNA), Rhodamine 123 (mitochondria) and Nile Red (plasma membrane) to study the cytotoxicity of these substances in human lymphoblastic cells sensitive (CEM-WT) or resistant (CEM-VLB and CEM-VM1) to anticancer agents. The results indicate a low resistance index of 2 for CEM-VLB cells treated with laxa B or laxa A + laxa B. For the three cell strains, following laxa B treatment, we observed an increase of a polyploid cell subpopulation that could result from the alteration of topoisomerase-II activity. On the contrary, the simultaneous treatment by laxa A and laxa B led to a decrease of that subpopulation with increasing laxa A doses. However, the effect of laxa A was less pronounced in the CEM-VLB cells, which present a low intrinsic topoisomerase-II activity. Though we observed a synergistic effect between laxa B and laxa A (the latter is inactive by itself), these results indicate a different mode of action for laxa B and laxa A + laxa B. A more precise study of the mode of action of these compounds is warranted.

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Laxaphycin A (laxa A) and Laxaphycin B (laxa B) were isolated from a terrestrial blue-green alga (cyanobacterium) Anabaena laxa (1) or from a marine cyanobacterium Lyngbya majuscula (2). They are cyclic peptides with antifungal and cytotoxic activities (2, 3). Although form A has no cytotoxic effect, form B possesses a significant cytotoxic effect inhibiting cell division and growth of human tumour cells, while the two forms (A+B) combined present different results depending on the ratio of the mixture, indicating a synergistic effect (1, 2). To learn more about the intracellular effects of these drugs, a protocol of multiple labelling was used (4-6). It involves the use of Hoescht 33342 (Ho342), a vital fluorescent dye specific for nuclei (7, 8) and of Rhodamine 123 (R123), specific for mitochondria (9-11). Nile Red (NR) is a fluorescent probe with a broad emission spectrum in the red region. Because of its high hydrophobicity, NR is rapidly trapped in the plasma membrane allowing a clear delineation of cell contour and, thus, the determination of the cell area (4, 11). This method was used to compare the effects of laxa A and laxa B on three established human lymphoblastic cell lines: CCRF-CEM-WT (sensitive), CCRF-CEM-VLB (“classic” MDR) (12, 13) and CCRF-CEM-VM1 (“atypical” MDR) (14, 15).

Materials and Methods

Cells culture. Experiments were performed on three established human lymphoblastic cell lines: parent drug-sensitive CEM-WT cells (WT for wild-type) and its multidrug resistance (MDR) derivatives CEM-VM1 and CEM-VLB. All the three cell lines were kindly provided by Dr. W. T. Beck (College of Medicine, University of Illinois, Chicago, IL, USA). CEM-VM1 cells had a stable resistance, while CEM-VLB cells were grown in culture medium with 100 ng/ml vinblastine in order to preserve their resistance, and further seeded in vinblastine-free medium 8 days prior to the experiments.
All the cells were grown as stationary suspension cultures in RPMI 1640 medium (Flow, Cergy Pontoise, France) supplemented with 10% heat-inactivated foetal calf serum (Gibco, Egny, France), 2 mM L-glutamine (ICN, Orsay, France) and 1% antibiotics stock solutions at 10,000 unit/ml penicillin and 10,000 μg/ml streptomycin, at 37°C in an atmosphere of 95% air and 5% of CO₂, in a humidified incubator (12, 13). The cells were seeded at 2x10⁵ cells/ml every four days in order to obtain a final density between 1 and 2x10⁶ cells/ml. In this way, the cells were used at an exponential growing phase. In all experiments, the viability of the cells was determined by the Trypan Blue exclusion test and was always higher than 97%. The cell lines were always free of mycoplasma contamination.

Cytotoxicity and kinetic growth assays. The growth-inhibitory effect of laxa A and laxa B, used alone or in combination, was assessed by plating cells in dishes (35 mm diameter) or flasks (25 cm²) at an initial density of 2x10⁶ cells/ml in a final volume of 1.5 ml for dishes and 6 ml for flasks. The cells were exposed to drugs for four days. The cytotoxicity of the laxaphycins, corresponding to a growth-inhibition test, on CEM-WT, CEM-VLB and CEM-VM1 cells was determined by counting the cell populations every day with a counter (model ZM, Beckman Coulter, Paris, France). The treatments were designated: C1 (laxa B 1 μM), C2 (laxa B 2 μM), C3 (laxa A 0.2 μM + laxa B 0.4 μM), C4 (laxa A 0.4 μM + laxa B 1 μM) and C5 (laxa A 1 μM + laxa B 1 μM). These treatments were compared to the control C0 (free from laxa A and laxa B).

The 50% inhibiting concentration (IC₅₀) was defined as the drug concentration that inhibited the cell growth by 50% with respect to untreated cells. The resistance index (RI) value of each MDR cell line, defined as the ratio of the IC₅₀ of CEM-VM1 or CEM-VLB to that of CEM-WT (control), was calculated. Afterwards, cells were labelled with specific stains, as described below.

Chemicals and staining procedure. Laxa A and B were extracted, purified and kindly provided as small crystals by the GEMMIB (Groupe d’Etude des Métabolites Marins d’Intérêt Biologique, Centre de Phytopharmacie, Université de Perpignan, France). They were kept in glass tubes at ambient temperature. Stock solutions of laxa A and laxa B were prepared in dimethylsulfoxide (DMSO; Merck, Nogent-sur-Marne, France) and stored at −20°C. Stock solutions of Ho342 (1 mM) (Aldrich, Saint Quentin Fallavier, France) and R123 (0.1 mM) (Sigma, Saint Quentin Fallavier, France) were prepared in phosphate buffer saline (PBS) (ICN) and kept at −20°C while NR (1 mM) (Sigma, Saint Quentin Fallavier, France) were prepared in phosphate buffer saline (PBS) (ICN) and kept at −20°C while NR (1 mM) was made up of laxa A and kept at 37°C. After the first hour of incubation, 115 μl of R123 were added to the cell suspension. The cells were further incubated for 57 min with the two probes before 15 μl of NR were added. After the remaining 3 min, the cell suspension was centrifuged and rinsed three times with 1 ml of Hank’s balanced salt solution (HBSS) (ICN). The cells were then suspended in 1 ml HBSS and 300 μl of that cell suspension were plated in Sykes Moore chambers for observation and numerical image multiparametric analysis.

Numerical image multiparametric analysis. The fluorescence digital imaging microscopy system (4, 16) consists of an inverted fluorescence microscope (IMT2, Olympus, Rungis, France) equipped with an epi-illuminator, a 40X objective (Leitz, Rueil-Malmaison) and a Silicon Intensified Target (SIT) camera (Lhesa, Saint Quentin, France) coupled to a Matrox MVP-AT digitizing card (Matrox, Dorval, Canada). Depending on the excitation wavelength required, a mercury or xenon lamp can be used. For the present experiments, a high-pressure mercury lamp (100 W) was used. The mercury lines at 365 nm and 435 nm were selected by dichroic mirrors to selectively excite Ho342 and R123 or NR. A computer controlled filter holder automatically stepped a filter depending on the specific fluorescence of the dyes: no filter for Ho342; an interference filter λmax = 525 nm, half bandwidth = 10 nm for R123; and an interference filter λmax = 610 nm, half bandwidth = 20 nm for NR. A specific data acquisition program (16), which included the background subtraction, the correction of non-linearity of the digitiser and the pixel-to-pixel heterogeneity of the camera gain, permitted the quantitative measurement of the dyes fluorescence intensities. The excitation intensity was adjusted with neutral density filters to keep each fluorescence probe image on a proper range of scale. A protocol was developed to analyse the fluorescence of the three stains: Ho342-stained nuclei, NR-stained plasma membrane and R123-stained mitochondria (4, 5). It allowed (17) the determination of cell and nucleus masks after image segmentation by thresholding, followed by a contour smoothing involving opening and closing sequences. The mask of nuclei or cells was used to evaluate nuclear and cellular parameters. Cell mask was also necessary to evaluate, by superimposition to the granular or punctuated image of R123-stained mitochondria, the mitochondrial parameters.

For each experiment, 22 parameters were evaluated including cell and nucleus area and perimeter, shape factors for the cell and the nucleus, and fluorescence intensity parameters (total, mean and standard error) for Ho342, R123 and NR on 200 to 750 cells.

About 30 min were necessary to acquire the images of the three stains for all cells and 10 min to analyse them. The fluorescence intensity parameters were normalised, using a reference standard, to compensate for mercury lamp fluctuations. First of all, cell size and shape factor were used to exclude cell clumps and debris and to keep only isolated cells. Afterwards, cell population properties were analysed through cell population distribution histograms for each of the selected parameters and cell distribution clouds in bivariate representation. The polyploidy of a cell population was evaluated through the percentage of cells presenting a Ho342 total fluorescence intensity (Ho342 [FI]) higher than twice the value of Ho342 [FI] for the G0-G1 peak obtained for control cell populations plus twice its standard error.

Results

Cytotoxicity. No cytotoxic or cytostatic effects were observed for laxa A up to 4 μM (data not shown). The cell growth curves obtained (Figure 1) exhibited different trends according to cell types and treatments. While the C2, C4 and C5 treatments fully blocked the cell growth for CEM-WT, only the C2 and C5 treatments did the same on CEM-VM1 cells. The C2 and C5 treatments blocked only 75% of the cell growth of CEM-VLB cells. In parallel, the C1 and C3 treatments, which induced a 50% inhibition of cell growth for CEM-WT, led to only 44% and 40% inhibition for CEM-VM1 and CEM-VLB, respectively. The CEM-V1

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cells appeared to be a little less sensitive than the CEM-WT cells. The CEM-VLB cells presented the characteristics of a low level of resistance with an IC$_{50}$ of 2 µM for laxa B and a resistance index value of 2.

Study of cell population properties. The cell cycle study through the DNA histograms (Figure 2) revealed a shift of the cell distribution toward high Ho342 tFI values compared to control cells. It appeared clearly that the C2 dose induced a complete blocking of the cell growth. That indicates, though the cell population growth is fully blocked, the capability of the cells to undergo DNA synthesis much far away from G2+M cell cycle phase. Thus, a subpopulation of polyploid cells appeared. On the contrary, for the C5 treatment, the cell population growth was also fully blocked, but the distribution of the cell
population remained similar to that observed for control cells. The cells seemed to be blocked in the cell cycle phase that they were at the time of treatment.

The plot of the nucleus area versus the cell area for the same cell populations (Figure 3) clearly showed that the C2 treatment induced a strong increase of the number of cells presenting larger nuclear sizes. This fact can be associated with the increase of the polyploid cell population observed following C2 treatment. On the contrary, the C5 treatment resulted in a stability of the cell and nucleus areas, compared to the control cell population.

The mean values of the parameters that characterised the cell populations according to cell types and treatments are presented in Table I. These values reflect the cell population distribution histograms. The mean values, which roughly account for distribution changes of the cell populations, are closely correlated to the presence of polyploid cells. The polyploid cell subpopulation was evaluated through the percentage of cells presenting a Ho342 tFI higher than 60,000 a.u. for CEM-WT, 50,000 a.u. for CEM-VM1 and 25,000 a.u. for CEM-VLB.

There was a good correlation between Ho342 tFI variations and the percentage of polyploid cells. For CEM-WT, this percentage increased with laxa B to reach 23% at 2 μM (C2), with complete blocking of the cell growth. The simultaneous treatment with laxa B and laxa A induced a decrease of the percentage of polyploid cells and Ho342 tFI values with increasing laxa A doses. A complete blocking of cell growth appeared in C4 treatment. Increasing the laxa A dose to 1 μM (C5) led to a drastic decrease of the values of the parameters near to that of control cells.

The behaviour described above for CEM-WT cells remained the same for CEM-VM1 cells, with an overall increase of Ho342 tFI values and of the percentage of polyploid cells for all C1, C2, C3 and C4 treatments. The decrease of polyploidy resulting from laxa A treatment was only observed for the higher dose of laxa A (C5) for which: a) the observed parameter values were near those of control cells and b) a complete blocking of cell growth was noticed. Thus, in contrast to the CEM-WT cells, laxa A seemed less efficient in decreasing the percentage of polyploid cells in the CEM-VM1 cell line.

Though to a lesser extent, we observed for CEM-VLB cells treated by mixed laxa A and laxa B (C3, C4, C5), a behaviour similar to that of the CEM-WT cells, i.e. a decrease of polyploid cells following increased laxa A concentrations. On the other hand, there was also an obvious decrease of R123 tFI and Ho342 tFI values for the CEM-VLB control cells versus the CEM-WT or CEM-VM1 control cell populations. This is representative of the MDR pattern expressed by CEM-VLB cells.

Discussion

In the search for new molecules with possible therapeutic interest, we tested Laxaphycins A and B on a human lymphoblastoid cell line CEM-WT and two resistant derivative strains CEM-VM1 (atypical resistance due to a lower intracellular topoisomerase-II activity) (14, 15) and CEM-VLB (MDR-resistant) (12, 13).

The study of growth curves has shown that CEM-VLB, which were reported to be as sensitive as CEM-WT to laxaphycins treatments (2), present a low resistance index value of 2. This index is, however, lower than the value of

Figure 3. Distribution of CEM-WT cell populations as a function of nucleus area and cell area for control cells and cells treated for 96 hours with different concentrations of mixed Laxaphycin A and Laxaphycin B.
11 obtained for CEM-VLB and adriamycin (5). Thus laxa A and laxa B still appear very interesting, since cells resistant to adriamycin are five times less resistant to laxa B or to laxa A + laxa B. These results also indicated that the two peptides acted synergistically to produce cytotoxic and cytostatic effects. It was shown that laxa A enhanced the activity of laxa B. These results confirm previous observations (1, 2).

The study of the properties of human lymphoblastoid cell populations following treatment with laxa A and laxa B was performed using a protocol involving triple labelling with vital dyes and multifluorescence image analysis. This method allowed the evaluation of morphological parameters (size, area and shape) as well as cellular parameters related to cell functions (cell membrane, nuclear and mitochondria labelling).

The analysis of parameter variations showed: (i) Laxa A and laxa B exhibit two modes of action on sensitive CEM-WT cells. Laxa B alone (2 ÌM) induced the appearance of a polyploid cell subpopulation concomitant with a complete blocking of cell growth. A similar phenomenon has already been reported for drugs such as bisdioxopiperazines (17-19), which block topoisomerase-II activity in the process of chromosome condensation and segregation, resulting in cessation of cell division and the generation of polyploid cells. On the contrary, simultaneous treatment of cells with laxa A and laxa B induced a complete blocking of cell growth for a lower dose of laxa B, without, however, increase of the polyploid cell subpopulation. Thus, though laxa A alone was inactive, it enhanced the cytotoxic effect of laxa B, but with a completely different mode of action.

(ii) The observations on the CEM-VM1 cells were similar to those on the CEM-WT cells regarding the polyploid cell subpopulation. However, while laxa A at doses as low as 0.2 ÌM caused a decrease of the percentage of polyploid cells in CEM-WT cells, in CEM-VM1 cells no such decrease occurred even at 0.4 ÌM. Laxa A doses as high as 1 ÌM were needed to observe a diminution of the polyploid cell subpopulation and distribution of the cell populations similar to that of the controls. This behaviour of CEM-VM1 cells could be due to their low intrinsic topoisomerase-II activity combined with alteration of topoisomerase-II activity by laxa B.

(iii) Although CEM-VLB cells are resistant, they showed a similar behaviour to CEM-WT cells, exhibiting an increase of the polyploid cell subpopulation with laxa B treatment and a decrease of that subpopulation with

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**Table I. Summary of the values of the parameters measured using image analysis and the percentage of polyploid cells according to CCRF-CEM cell types and laxaphycin treatments.**

<table>
<thead>
<tr>
<th>CCRF-CEM Cell types and treatments</th>
<th>Parameters</th>
<th>Polyploid cells (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Cell area* (Mean)</td>
<td>NR tFI** (Mean)</td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C0 589 26733 8694 406 27277 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1 564 28205 9775 410 36626 12.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2 688 45175 22598 644 44275 23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3 655 62134 20103 547 32777 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4 458 40991 11393 406 29629 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C5 441 35800 10054 377 29497 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VM1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C0 505 30324 6750 406 21120 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1 552 41343 10736 452 29288 11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2 537 50176 13394 461 25632 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3 528 40188 21251 449 35400 15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4 551 38878 30114 415 35787 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C5 528 42956 12750 454 26863 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLB</td>
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<tr>
<td>C0 512 34106 1608 378 11773 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1 585 35262 1139 426 9745 2</td>
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</tr>
<tr>
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<tr>
<td>C3 553 34441 1485 398 10929 2</td>
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</tr>
<tr>
<td>C4 603 30886 877 415 7797 1</td>
<td></td>
<td></td>
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<tr>
<td>C5 531 58445 4135 434 13527 8</td>
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* all the values are given in pixels
** all the values are given in arbitrary unit [a.u.]; tFI: total fluorescence intensity; NR: Nile Red; R123: Rhodamine 123; Ho342: Hoescht 33342
simultaneous treatment with both peptides. The lesser effect of laxa B can be attributed to its lower intracellular accumulation, resulting from P-glycoprotein efflux.

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References

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