Bistramide A-induced irreversible arrest of cell proliferation in a non-small-cell bronchopulmonary carcinoma is similar to induction of terminal maturation

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Summary - Bistramide A, a new toxin isolated from a New Caledonian Urochordata, shows an antiproliferative effect on a non-small-cell lung carcinoma line in vitro and G1-blockade. In this work, the growth arrest induced by bistramide A was shown to be irreversible as assessed by growth kinetics of pretreated cells. Furthermore, the drug caused an underexpression of the nuclear antigen Ki67. These events are similar to a G1-differentiation cell cycle step blockage and a terminal maturation induction.

bistramide / terminal maturation / non-small-cell lung carcinoma / Ki67 antigen / irreversible growth arrest

Introduction

We previously studied bistramide A [14], a toxin from the marine invertebrate Lissoclinum bistratum Sluitter [4, 7], determining that its antiproliferative effect against a lung carcinoma cell line resulted from blockage of G1 phase cells associated with polyploidy due to an incapacity for cytodieresis [14]. The purpose of the present work was to determine whether this arrest of cell proliferation was reversible once the drug was removed from culture medium. In particular, we considered the effect of the drug on cell expression of a nuclear antigen (Ki67), which is expressed after the end of G1 phase and then continually during the S and G2M phases of proliferating cell cycle [5, 6]. This antigen is not expressed in normal quiescent cells [12], and is one from the pool of proteins with a short half-life stabilized in cancer cells [13].

Materials and methods

Bistramide A

Bistramide A (molecular mass 704 kDa) was isolated from the New Caledonian Urochordata Lissoclinum bistratum, as previously described [7]. Aliquots (1 mg/ml) were dissolved in 100 µl DMSO and 900 µl sterile water. Further dilutions were done in RPMI medium to give DMSO concentrations of less than 0.1% which had no effect on cells.

Cell line and cell culture

Clone L ×¢, from the NSCLCN ×¢, line was derived from a primary culture of moderately differentiated, rarely keratinized human non-small-cell lung carcinoma (classified T,N,M ×) [8]. Cells were cultured in RPMI medium supplemented with 5% fetal calf serum, 100 IU penicillin/ml, 100 µg/ml streptomycin and 2 mM glutamine. Cells used in all experiments never exceeded 35 passages.

Growth kinetics after treatment

Cells were incubated 72 h in 75 cm² flasks at a concentration of 2 × 10⁵ cells/ml in the culture conditions described above in the presence or absence of 0.21 and 0.07 µM bistramide A. After medium removal, cells were washed twice with Dulbecco's phosphate-buffered saline (D-PBS) and then dropped into 96-well microplates at a concentration of 2 × 10⁴ viable cells/ml (viability assessed by trypan blue dye exclusion) in fresh medium containing no drug. Cell growth was evaluated on this day (considered as day 0) and then at days 1, 2, and 3 by the colorimetric assay of Mosmann [11] using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduced to blue-black formazan by mitochondrial dehydrogenases of viable cells exclusively.

Flow Cytometric assays

Cell culture

Cells in exponential growth phase (24 h culture in fresh medium) at a concentration of 1.5 × 10⁵ cells/ml were cultured with or without 0.21 µM bistramide A for 1, 2 and 3 days without medium removal.

Double staining Ki67/DNA

Cells were stained using the washless double-staining method for Ki67 antigen and DNA [10]. Briefly, after medium removal, cells were washed twice with D-PBS 1% bovine serum albumin) and then incubated in 500 µl of lysis/DNA-staining solution (propidium iodide 20 µg/ml, RNase 0.2 mg/ml; Nonidet P-40 0.5% v/v and EDTA 0.5 mM in calcium- and magnesium-free PBS, pH 7.2) for 15 min. 10 µl of fluorescein isothiocyanate (FITC)-conjugated monoclonal Ki67 antibody was added for 30 min

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(DAKO F-788) to 250 μl of the cell suspension. An FITC-conjugated monoclonal anti-CD3 (Immunotech) was used as an irrelevant isotype control.

**FCM analysis**

Nuclei were analyzed with an ATC 3000 cell sorter (Bruker, Wissembourg, France) using 488-nm argon laser excitation (400 mW) and dual parameter acquisition of log green fluorescence through a BP 515 nm filter and linear red fluorescence through an LP 650 nm filter. Fluorescence signals were not electronically compensated. To eliminate doublets, nuclei for bivariate DNA/Ki67 analysis were selected by gating on the cytogram ‘DNA peak vs DNA area’. Each analysis was carried out on at least 30000 events.

**Results**

To study the irreversibility of bistramide A-induced arrest of cell proliferation, cells were pretreated for 72 h and then subcultured in fresh medium containing no drug (fig 1). Non-treated cells showed a classical kinetic pattern: growth phase during 24 h followed by slowing to a plateau phase. Cells treated with 0.07 μM of bistramide A were capable of proliferating in a drug-free medium even when their growth was slowed by the treatment. Cells treated with 0.21 μM of bistramide A did not enter into exponential growth phase. Their number remained stable for 24 h and then decreased; natural cell mortality was not offset by renewal of the cell population. The viability of these cells was demonstrated by formazan crystal formation by mitochondrial dehydrogenases [11] during the experiment and initially by trypan blue dye exclusion assay (more than 95% viable cells were put on the plates).

The visualization of the two-parameter contour plot for Ki67 expression and DNA content (fig 2) enabled us to separate two subpopulations among the non-treated G0/G1 cells after 72 h of culture: Ki67-positive cells (above) and Ki67-negative cells (below).

To study the influence of culture time in the same medium on G0 cells appearance, untreated cells cultured for 48, 72 and 96 h in the same drug-free medium were stained for Ki67 and DNA (fig 3). Mathematical processing of red

![Fig 1. Growth kinetics of the NSCLCN6L16 cells pretreated with: 0.07 μM bistramide A (★); 0.21 μM bistramide A (■); control (●). After 72 h of treatment with bistramide A at the indicated doses or in drug-free medium for the control, cells were plated in fresh medium containing no drug and were counted every 24 h (see Materials and methods).](image)

![Fig 2. Two-parameter contour plot for Ki67 expression (y axis, log scale) and DNA content (x axis, linear scale) in the untreated NSCLCN6L16 cells (arbitrary units). Because the difference between true G0 cells and G1-arrested cells in stringent physiological conditions is as for yet unclear [2], we have decided to call all Ki67 negative cells with the general term G0 cells in the figures.](image)

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<table>
<thead>
<tr>
<th>Sample</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>77</td>
<td>44</td>
<td>46</td>
</tr>
<tr>
<td>Treated</td>
<td>42</td>
<td>32</td>
<td>13</td>
</tr>
</tbody>
</table>

*Table 1. Percentage of Ki67-positive cells for untreated cells (Control) and cells treated with bistramide A 0.21 μM (Treated) at different times.*

fluorescence histograms with propidium iodide showed an increase in the proportion of cells with a DNA G0/G1 content during culture time. Biparametric cytograms of DNA content versus Ki67 antigen expression showed the appearance of a cell subpopulation with a DNA G0/G1 content and no Ki67 expression. Because these latter are considered as non-proliferating cells, quiescent or G1-arrested [6], double labelling with Ki67 versus DNA revealed the G0 phase cells.

To assess the ability of bistramide A on putting cells out of the cycle even in growth-promoting culture conditions, a study comparing double staining Ki67/DNA as a function of culture time for untreated cells and cells treated with bistramide A 0.21 μM is shown in figure 4. Treatment was started after 24 h of culture. Figures 4A, B, C concern the populations treated with 0.21 μM of bistramide A for 24, 48 and 72 h, and figures 4D, E, F the simultaneously harvested, untreated populations treated for 48, 72 and 96 h in the same medium. The tridimensional presentation of the DNA vs Ki67 cytograms indicates a progressive decrease of the number of cells in cycle phases (Ki67-positive S, G2M and G1 cells) for those populations treated with bistramide A, whereas a high proportion of Ki67-positive cells remained in the controls despite impoverishment of the medium.

The percentages of Ki67-positive cells for every sample are given in table 1.
Bistramide A-induced terminal maturation

Fig 3. Bivariate Ki67 Ag/DNA cytograms (right) and Ki67 histograms (left) of untreated cells. The histograms show the appearance of unfluorescent (Ki67 negative) cells. Arbitrary units. A, 48 h culture. B, 72 h culture. C, 96 h culture. The Ki67-negative cells are called GO cells for commodity (see fig 2).

Discussion

We previously demonstrated that bistramide A has an antiproliferative activity on the NSCLCN6-L16 cell line [14] due to G1 blockade and an inaptitude for cytodieresis. The blockade is dose- and time-dependent. Beyond 48 h, this antiproliferative effect leads to progressive cell mortality and an ID50 in the order of 0.49 μM. Craig et al [1] showed that the irreversible blockade of a human myeloblastic leukemia cell line in G1 phase occurred only when intercalating agents in the presence of differentiation factors led to an increase in the number of differentiated cells. Growth resumed when the drug was removed in the absence of differentiating factors. Moreover, Wille and Scott [15] found that differentiation could be induced in 3T3 mesenchymal cells arrested at a certain point of the G1 phase, referred to as a G1-differentiation (G1-D) stage. This G1-D point is topographically (and chronologically) distinct from the ones of the G1 phase where the cells are blocked when they are serum- or nutrient-deprived [16]. From this G1-D point, cells can, depending on the environmental conditions, re-enter the cell cycle to proliferate normally or enter the differentiation process. The different steps of this maturation are, in this order: reversible proliferation arrest; differentiated phenotype acquisition; and finally the irreversible loss of proliferation capacity that is the mark of terminal maturation [3].

Two cell subpopulations with the same DNA G1 content were differentiated by analysis of Ki67 nuclear antigen expression on mononuclear leucocytes in peripheral blood stimulated by phytohemagglutinin (PHA) [6]. Without stimulation, only a single weakly labeled G1 cell population was found, considered to be a delayed or differentiated G0/G1 phase. Antigen Ki67 expression increased as a function of PHA stimulation time. Disappearance of Ki67 antigen was noted when proliferating cells were pushed toward maturation [6]. Further analysis showed that Ki67 antigen appeared at a precise stage of the G1 phase of the cell cycle [5]. The Ki67 antigen is not expressed in normal quiescent cells when it is still expressed in cancer cells arrested in G1 in case of lack of nutrients [9]. This protein is one of the pool of molecules with a short half-life that are stabilized in cancer cells [13], whose precise function in the regulation of proliferation is actually investigated [12, 13].

In the present work, we have noted that the increase in the proportion of non-proliferating cells caused by the impoverishment of culture medium is assessed by Ki67 antigen expression. After 48 h, when the cells were in exponential growth phase, the proportion of Ki67-positive cells was estimated at 77% (table 1), but after 72 and 96 h of culture this percentage decreased by half due to an increase in the number of G0 or G1 phase cells delayed by an impoverishment of medium growth factors in these culture conditions (fig 3A, C. table 1).

Whereas impoverishment of culture medium caused a moderate disappearance of non-proliferating cells, treatment with 0.21 μM bistramide A resulted in almost complete disappearance of proliferating cells (fig 4C). This cell pool was composed mainly of cells in the G0 or G1-delayed or in the G1-D cell cycle phase, according to references [6, 15].

The inhibition of cell proliferation caused by nutrient deprivation or serum starvation is reversible, whereas terminal maturation reached through the G1-D cell cycle phase is characterized especially by the irreversibility of the arrest in cell growth [3].

As shown by figure 1, even when they are put in fresh medium sufficient to allow untreated cells to proliferate, cell populations treated with bistramide A at a dose of 0.21 μM have lost their growth capacity. More than 50% of these cells die, and those that remain alive do not express Ki67 antigen.

Progressive blockade of the cell population in G0/G1 phase due to the effect of bistramide A, in association with decreased Ki67 expression, indicative of a living but non-proliferating cell population, and irreversible arrest of proliferation, is similar to the entry of cells in G1-D phase and to induction of terminal maturation.
Fig 4. 3D-bivariate Ki67 Ag/DNA cytograms, featuring controls on the right and samples treated with 0.21 μM bistramide A on the left, at different times. Treatment was started after 24 h of culture. A, D. 24 h treatment and 48 h culture. B, E. 48 h treatment and 72 h culture. C, F. 72 h treatment and 96 h culture.

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