Diverse effects of the cyclin-dependent kinase inhibitor bohemine: Concentration- and time-dependent suppression or stimulation of hybridoma culture

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Received 18 December 2000; accepted 30 April 2001

Key words: cell cycle, fed-batch culture, hybridoma, purine derivatives, semicontinuous culture

Abstract

An analog of aromatic cytokinins, the 2,6,9-trisubstituted purine derivative bohemine, was applied to cultures of mouse hybridoma cells in order to analyze its capacity of suppressing cell growth and maintaining or enhancing the production of monoclonal antibody. Addition of bohemine at concentrations in the range of 1–10 μ M resulted in a short-term arrest of growth and of monoclonal antibody production. The short-term suppression of cell functions was followed by a significant temporary increase of specific growth rate and of specific production rate. The steady-state viable cell density values, found in semicontinuous cultures, showed a certain stimulation of cell growth in the range of micromolar concentrations of bohemine, and inhibition of growth at 10 and 30 μ M concentrations. The profiles of cell cycle phases indicated that hybridoma cells are retarded both at the G₁/S boundary and at the G₂/M boundary, depending on bohemine concentration. The existence of the sequence of events, from suppression to stimulation, suggests that bohemine probably modulates more than one regulatory pathway in the cell.

Abbreviations: MAb, monoclonal antibody; CDK, cyclin-dependent kinase.

Introduction

Suppression of cell growth has been found to be accompanied frequently with enhanced production of the secreted protein (Suzuki and Ollis, 1990; Suzuki et al., 1992; Takahashi et al., 1994). Therefore, slowing down the cell cycle progression while maintaining the cells in viable state is one of the important tasks in development of new efficient technologies employing animal cell lines. Apart from genetic modifications of the cell line (Kirchhoff et al., 1996; Fussenegger et al., 1997), manipulation with the cell growth rate by providing external molecular signals constitutes a means how to prolong the lifespan of cultured cells and to increase the yield of the secreted protein. Addition of butyrate represents a classical example of

cell growth modulation by a low-molecular substance (Kruh, 1982).

In our previous work we examined the possibility of cell growth suppression by application of an acyclic nucleotide analog 9-[2-(phosphonomethoxy)ethyl]-guanine. This substance is known to cause transient inhibition of DNA synthesis (Holý, 1994; Kramata et al., 1996). Significant suppression of hybridoma growth could be observed at remarkably low concentrations, namely 10 to 100 nM (Franěk et al., 1998). Concomitantly with the reduction of the growth rate, the specific MAb production rate rose by 20–27%.

In the present work we focused our attention to another group of synthetic antiproliferative substances possessing the capacity of perturbing the progression of the cell cycle through inhibition of cyclin-dependent

kinases (CDKs). Typical low-molecular inhibitors of CDKs are synthetic analogs of aromatic cytokinins, i.e. of plant hormones, namely 2,6,9-trisubstituted purine derivatives, olomoucine and roscovitine. The mechanism of action of these substances has been recognized as the competition with ATP binding to the CDKs (Veselý et al., 1994; Pines, 1995; DeAzevedo et al., 1997; Hajdúch et al., 1999). The potential of purine derivatives as chemotherapeutic agents, specifically new anti-cancer drugs, provoked the recent effort at synthesizing hundreds of new derivatives (Legraverend et al., 1999, 2000; Hajdúch et al., 1999; Chang et al., 1999; Otyepka et al., 2000).

Antiproliferative substances regularly induce apoptosis, depending on the concentration applied. Apoptosis, desirable in cancer therapy, is undesirable, of course, in the production culture. One of the new derivatives, bohemine, has been found, in our pilot studies on hybridoma cultures, to be devoid of apoptosis-inducing activity at concentrations up to 30 μ M. This finding prompted us to undertake a more profound study of the effect of this substance on hybridoma cells cultured under various culture modes. We found, surprisingly, that a short-term suppression of cell functions, occurring upon application of bohemine, was followed by a significant increase of specific growth rate and specific production rate.

Materials and methods

Cell line and media

Mouse hybridoma ME-750 was cultured in DMEM/F12/RPMI 1640 (2:1:1) medium supplemented with BME amino acids, 2 mM glutamine, 0.4 mM each of alanine, serine, asparagine and proline (Franěk and Šrámková, 1996), 15 mM HEPES, 2.0 g l⁻¹ sodium bicarbonate and with the iron-rich protein-free growth-promoting mixture (Franěk et al., 1992).

Cell culture

Static cultures and semicontinuous cultures were set up in a total volume 6.0 ml in 25 cm² T-flasks. In fed batch cultures, a volume of 0.3 ml of medium, containing essential amino acids, vitamins and glucose, was added daily starting from day 1. The assays starting at high cell density were set up using a cell inoculum enriched by centrifugation.

In semicontinuous cultures a volume of 2.0 ml was withdrawn once per day, and replaced by 2.0 ml of me-

dium or by medium containing the tested substance. The values of cell concentrations and of MAb concentrations were recorded daily. At least five values were averaged to obtain steady-state mean values.

Stirred cultures were carried out in spinner flasks in a culture volume of 200 ml. All cultures were kept at 37 °C in a humidified atmosphere of 5% CO₂/95% air.

Viable cells and dead cell were counted in a hemocytometer using trypan blue exclusion test. The experimental error involved in the estimation of cell density was $\pm 10\%$. The MAb concentration was determined by immunoturbidimetry in two parallels for each sample (Fenge et al., 1991). The experimental error associated with the estimation of MAb concentrations was $\pm 5\%$.

Cell cycle profiles

The cells ($5\text{--}10 \times 10^6$ ml⁻¹) were permeabilized and stained with propidium iodide using DNA Prep Reagent Kit (Coulter Immunology, Hialeah, FL) according to manufacturer's protocol. The samples were measured with EPICS Profile II flow cytometer (Coulter Immunology, Hialeah, FL) and data were analyzed by Multicycle software (Phoenix Flow Software Products, San Diego, CA). For the determination of the cell cycle profiles in semicontinuous cultures at least three samples taken at various days were analyzed after establishment of the steady-state. Percentage of cells in G₀/G₁, S, and G₂/M phases was evaluated. The content of cells with subdiploid content of DNA, representing apoptotic cells with partly fragmented DNA, was expressed as percentage of total G₀/G₁+S+G₂/M cells.

Chemicals

Powdered media were from Life Technologies. All added chemicals were of analytical grade purity.

Bohemine (2-[(3-hydroxypropyl)amino]-6-benzylamino-9-isopropylpurine) was synthesized in the Isotope Laboratory of the Institute of Experimental Botany (Praha) according to published procedures (Havlíček et al., 1997; Otyepka et al., 2000). Stock solution (10 mM) of bohemine was prepared in dimethylsulfoxide. Working dilutions were made in culture medium.

Results

Growth suppression potential of bohemine

Graded concentrations of bohemine, from 1 μM to 30 μM , were added to culture media in a pilot series of assays set up in 25 cm^2 T-flasks. Viable and dead cells were counted on culture days 3 and 6. In the presence of 10 μM and 30 μM bohemine the viable cell count was significantly lower with respect to control, i.e., 77% and 48%, respectively. When tested after 6 day culture period, the cell viability in individual assays was in the range of 70–80%, even at the highest bohemine concentrations tested. These results confirmed the mild character of bohemine action, and made it possible to define the range of concentrations, at which the apoptosis inducing potential of this compound could be neglected.

Growth kinetics upon addition of bohemine

The changes of culture parameters accompanying the addition of bohemine in the course of hybridoma batch cultures were examined in stirred cultures. In a series of experiments bohemine was added, at various times during the exponential phase, to final concentrations in the range of 1 to 10 μM . In all cases a drop of specific growth rate and of specific MAb production rate was observed within 24 hr upon bohemine application. However, during next one or two days both the growth rate and the production rate recovered, and, in most cases, even exceeded the values of the control experiment.

Two characteristic examples are presented in Figure 1. The application of a relatively high bohemine dose on day 1 (see the left panel) resulted in a growth arrest lasting for about one day. A significant drop in MAb production lasted for about two days. An intense enhancement of growth and production rates followed the period of general suppression. The viability of the bohemine supplemented culture on day 8 was higher than the viability in the control culture. The addition of a lower bohemine dose on day 2 (see the right panel) caused also a growth arrest and a drop in the specific MAb production rate. Similarly with the preceeding experiment the specific production rate temporarily acquired a considerably high value. The growth rate recovered, but did not exceed significantly the control value. Final MAb concentration was in this experiment higher than in the control culture by about 10%.

Table 1. Final MAb concentrations in cultures supplemented with bohemine^a

Bohemine (μM)	MAb concentration (mg l^{-1}) ^b	
	Batch mode	Fed batch mode
0 (Control)	47 $\pm$ 11	152 $\pm$ 17
1	49 $\pm$ 12	161 $\pm$ 18
3	60 $\pm$ 13	180 $\pm$ 18
10	46 $\pm$ 12	143 $\pm$ 17
30	46 $\pm$ 9	93 $\pm$ 14

^a Data from experiment depicted in Figure 2.

^b Mean $\pm$ SD.

The effect of bohemine application to cultures approaching the stationary phase was examined in a series of assays set up at starting cell density of about 1×10^6 cells ml^{-1} . Two sets of assays in 25 cm^2 T flasks were started from the same inoculum. One set of assays was conducted in the batch mode, the other set in fed batch mode (Figure 2). Except for the highest concentrations, 10 and 30 μM , bohemine did not suppress significantly the cell growth up to day 3. In the period beginning on day 5 the viable cell densities at low bohemine concentrations were slightly higher than the cell densities in the control assay. These characteristic changes were even more pronounced in the set of assays carried out in fed batch mode. The final MAb concentrations in the batch mode set differed only negligibly from the control (Table 1). The MAb concentrations in the fed batch mode set were generally markedly higher. Clearly, bohemine at 30 μM concentration inhibited not only the growth, but also the MAb synthesis. The increase of MAb concentration by about 18%, relative to control, at 3 μM bohemine did not reach the level of significance ($P < 0.05$).

Bohemine in semicontinuous cultures

The aim was to investigate the character of bohemine action in a culture mode in which the concentration of the tested substance is maintained at constant level for a long time period. An insignificant increase of viable cell density was observed at bohemine concentrations in the micromolar range, while significantly lower values were found at bohemine concentrations 10 and 30 μM (Figure 3). The same trend could be observed with the steady-state MAb concentrations.

The cell cycle profiles showed two kinds of concentration-dependent changes. The major trend was the increase of G_0/G_1 phase fraction at the expenses of the S phase fraction, in parallel with increas-

Figure 1. Stirred batch cultures of hybridoma ME-750 treated with a single dose of bohemin. Left panel: 10 μ M bohemin added on day 1. Right panel: 2 μ M bohemin added on day 2. Open symbols: Untreated control. Closed symbols: Culture with bohemin addition.

ing bohemin concentration. This change pointed to an existence of a block in the G_1 to S phase transition. A minor concentration-dependent change consisted in an increase of G_2/M phase cells in 3 and 1 μ M bohemin-containing media. The minor change indicated a retardation of the G_2 to M phase transition. The cell cycle analysis showed also a relatively low and constant level of apoptotic cells in the whole range of bohemin concentrations tested.

Discussion

Synthetic inhibitors of CDKs are under most serious consideration as new anti-cancer drugs. The development of new analogs, and the studies on their mechanism of action, have recently acquired increased speed (Hajdúch et al., 1999). One of the classes of CDK in-

hibitors are cytokinin analogs, derived from the lead compound olomoucine (Veselý et al., 1994). These derivatives have been shown to inhibit several CDKs, and to arrest cells in both G_1/S and G_2/M boundaries (Meier et al., 1997).

One of the new derivatives, bohemin, currently underwent preclinical studies as an anti-cancer drug. In another application, bohemin served as a kinase inhibitor in the activation of bovine oocytes. Inhibition of DNA synthesis in oocytes was shown to be reversible, because after removal from bohemin-supplemented medium the activated oocytes started to synthesize DNA (Alberio et al., 2000). An attempt at elucidating the molecular basis of bohemin activity was undertaken through employing proteomics technology. T-lymphoblastic cell line CEM was treated by bohemin and the changes of the protein composition, as visualized in two-dimensional electrophoresis, were

Figure 2. Static cultures of hybridoma ME-750 treated with various doses of boheminine. Assays initiated with cell-enriched inoculum at high cell density. Boheminine was added to the media at inoculation.

quantitated. In boheminine treated cells, five proteins were found to be downregulated, namely α -enolase, triosephosphate isomerase, initiation factor 5A, and α - and β -subunits of Rho GDP-dissociation inhibitor 1 (Kovářová et al., 2000). These proteins play significant roles in glycolysis, proteosynthesis, and in cytoskeleton rearrangement. This finding shows that the cellular effects of boheminine could be mediated by a variety of factors in addition to CDK inhibition.

Another 2,6,9-trisubstituted purine, myoseverin, was found to induce reversible fission of multinuclear myotubes. Upon removal of the compound, DNA synthesis and cell proliferation started with higher intensity than in the untreated control (Rosania et al., 2000). A number of 93 genes was found to be significantly affected by the compound. On the other hand, no inhibition of CDKs was reported. All these findings obtained in other cell systems add weight to the view that the complex character of boheminine action, as observed in our work, is an expression of the pleiotropic fashion how boheminine addresses cellular targets.

The specific features of the complex response of hybridoma cells to a single dose of boheminine (Figure 1) are (i) the spontaneous resumption of functions,

starting after a short period of arrest of functions, even if the agent is not removed, and (ii) the high peak values of the specific growth and production rates in the resumption phase that, in most cases, exceed the corresponding values in the untreated control. The mechanism of action of boheminine needs further elucidation. The high peak values of specific rates, appearing in the resumption phase, provide a strong quantitative argument against a possible view, namely that boheminine is simply decomposed in the cell, and the cell returns to its normal state. The switch from suppression to stimulation may be caused by enzymic alteration of boheminine in the cells. The product(s) of the reaction need not be neutral metabolites, and may elicit different responses. Alternatively, the cellular stress induced by boheminine might provoke an anti-stress reaction resulting in temporarily intensified cellular processes.

In a recent work (Chmela et al., 2001) the *in vivo* metabolism of radiolabeled boheminine in mice was investigated. The terminal hydroxyl group located at the C2 side of boheminine was found susceptible to various metabolic attacks. Two glycosidation products were identified, namely boheminine O- β -D glucoside and bo-

Figure 3. Semicontinuous cultures of hybridoma ME-750 in the presence of various bohemine concentrations. Steady-state values of viable cell density, MAb concentration, distribution of cell cycle phases, and estimated fraction of apoptotic cells are given.

hemine β -glucuronide. Another principal bohemine metabolite was a carboxylic acid, 6-benzylamino-2-(2-carboxyethylamino)-9-isopropylpurine, appearing in the cytosol as a product of oxidation catalyzed by alcohol dehydrogenase. In future studies the effects of these metabolites on cultured cells have to be elucidated.

The analysis of bohemine application to high-density cultures (Figure 2) was carried out with the aim to define the range of low initial bohemine concentrations, in which the stimulatory activity might override the inhibitory activity of the compound. The success was only partial. At certain low initial bohemine concentrations we were able to prolong a little bit the lifespan of the culture. The enhancement of final MAb yields in bohemine treated cultures, however, was hardly significant from the point of view of MAb production, even in the fed batch made of culture.

To complement the knowledge on bohemine activities, we have set up semicontinuous cultures in the presence of various bohemine concentrations. The semicontinuous culture, based on the chemostat mode of cell culture, enables establishment of steady-states at which the inhibitory and stimulatory forces are in balance. The steady-state cell cycle profiles reflected both the block in G_1/S phase transition and the block in the G_2/M phase transition. Both these blocks could be expected from findings obtained with similar compounds on other cell lines (Meijer et al., 1997). In our system the blocks or retardations in the phase transitions could be partly separated (see Figure 3). The relative accumulation of G_2/M phase cells was highest at 3 μM bohemine, while the G_0/G_1 phase cells showed an increase up to 30 μM bohemine.

The present study of bohemine action on hybridoma cultures is the first application of a compound

from the 2,6,9-trisubstituted purines library in a biotechnological process. We believe that the results of this analysis will help, in the future, to select other analogs more suited for the purpose of intensifying protein production by cultured animal cells.

Acknowledgements

This work was supported by the Grant 303/99/1541 from the Grant Agency of the Czech Republic, and by the Grant 111300005 from the Ministry of Education, Youth and Sports of the Czech Republic. Qualified assistance of Ludmila Bordeová is highly appreciated.

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