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Short Communication

High-performance liquid chromatographic monitoring of metabolic products resulting from the treatment of mouse hepatoma cells with N^6-cycloalkylated nucleosides

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ABSTRACT

High-performance liquid chromatography was used to analyze cell lysates and growth medium of mouse hepatoma cells, separately treated with N^6-cyclopropyl-, N^6-cyclobutyl-, and N^6-cyclopentyladenosines, in an effort to gain insight into the mechanism by which these modified nucleosides exert their cytotoxic effect(s). The corresponding 5′-monophosphate of the respective modified nucleoside was detected in the separate cell lysate samples. Both the modified nucleoside and its corresponding 5′-monophosphate were detected in the separate growth medium samples and their relative concentrations therein were determined. These results indicate that the cytotoxicity of these N^6-cycloalkylated nucleosides may be attributed to their 5′-monophosphates within the cells.

INTRODUCTION

Previous studies have shown that when certain mammalian cells were treated with synthetic nucleic acid derivatives, those modified bases and/or nucleosides that were substrates for adenine phosphoribosyl transferase and adenosine kinase were converted to the corresponding 5′-monophosphates [1]. It has been established that the cytotoxicity, associated with alkylated bases and nucleosides, is dependent upon their conversions to the 5′-monophosphates [2].

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The modified nucleosides, N^6-cyclopropyl-, N^6-cyclobutyl-, and N^6-cyclopentyladenosines, were chosen for the present studies since it had been recently reported that they were more cytotoxic to mouse hepatoma cells than the N^6-cyclopropyl-, N^6-cyclobutyl-, and N^6-cyclopentyladenines [3]. It was anticipated that the sensitivity and rapidity of high-performance liquid chromatographic (HPLC) techniques [4] could be utilized to detect the presence and quantify the concentrations of metabolic products resulting from the treatment of mouse hepatoma cells with these modified derivatives, by analyzing the cell lysates, as well as the medium in which the treated cells were grown. Furthermore, it was anticipated that some insight into the mechanism, by

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which these compounds exerted their cytotoxic effect(s), could be ascertained.

EXPERIMENTAL

Apparatus

A Beckman Model 331 isocratic HPLC system (Beckman Instruments, Fullerton, CA, USA) equipped with a UV 160 absorbance detector with a fixed wavelength at 254 nm was employed.

Chromatographic conditions

A Beckman Ultrasphere ODS pre-packed, reversed-phase column (5 μm, 25 cm × 4.6 mm I.D.) equipped with a guard column (5 μm, 4.5 cm × 4.6 mm I.D.) was used (Beckman Instruments). The operating pressure varied between 13.8 and 20.7 MPa and the elution profiles were recorded at a chart speed of 2.51 cm/min, providing retention times with a standard deviation of 0.02. A methanol–water (70:30, v/v) solution, at a flow-rate of 0.8 ml/min, was utilized for all elutions.

Chemicals

HPLC-grade methanol and water, used to prepare the solvent system, were purchased from Fisher Scientific (Norcross, GA, USA) and were filtered through a 0.2-μm PTFE membrane before use (Lazar Scientific, Los Angeles CA, USA). Cyclic AMP was purchased from Sigma (St. Louis, MO, USA).

Standard procedures were utilized to synthesize the modified nucleosides and their corresponding 5′-monophosphates [5]. These included N^6-cyclopropyl-, N^6-cyclobutyl-, and N^6-cyclopentyladenosines and their 5′-monophosphates, respectively. The purity (97–98%) of each derivative was determined by paper chromatography in several solvent systems and ultraviolet spectroscopy on a Beckman DU-7HS spectrophotometer.

Cell line

A subclone (HEPA-2) of BW7756 mouse hepatoma cells was used in these studies. The cells were grown in Dulbecco's Modified Eagles' Medium (DMEM) and routinely maintained in the log phase of growth by subculturing them weekly in 74-cm^2 tissue culture flasks. The DMEM was prepared from a ready-mixed preparation from Sigma. The medium was supplemented with 3.5 g/l glucose, 3.7 g/l bicarbonate, 10% fetal bovine serum (Sigma), 100 U/ml penicillin, and 100 g/ml streptomycin. The cells were grown in a humidified atmosphere of 5% CO_2 at 37°C. Under experimental conditions, 90–95% of the attached cells continuously showed characteristic growth patterns of healthy hepatoma cells.

Experiments and sample preparation

The experimental cultures were prepared, from a stock of single cell suspension, by trypsinizing the confluent cells with 3 ml of 10% trypsin and adding the suspension to 7 ml of DMEM. Portions (1 ml) of the cell suspension (10^6 cells), determined by hemocytometry, were placed into separate 60-mm^2 plastic tissue culture flasks and inoculated with individual samples (3 ml, 33 μg/ml) of each N^6-cycloalkylated nucleoside dissolved in 0.2 *M* Tris–maleate (pH 7.2). The separate flasks of treated cells were incubated, together with a control, at 37°C for 24 and 48 h, during which time a 1-ml sample of the growth medium was removed from each flask, at the end of incubation period, for subsequent analysis by HPLC. After each incubation period, the treated cells were collected by centrifugation (2930 *g*, 10 min). The resulting cell pellet was resuspended in fresh DMEM (2 ml) and recentrifuged (2930 *g*, 10 min). Finally, the pellet was lysed with 0.5% Triton X-100 in 0.13 m*M* sodium chloride, 5 m*M* potassium chloride, and 7.4 m*M* magnesium chloride. After centrifugation (2930 *g*, 10 min), the supernatant was filtered through an ultrafiltration cone (Amicon Type CF-25) to remove proteins and other macromolecules. Aliquots (10 μl) of the lysates, as well as the previously removed 1-ml samples of growth medium, in which the cells were grown, were analyzed by HPLC as described below.

Calibration and peak identification

Standard solutions of each N^6-cycloalkylated

derivative were prepared at 0.1 mg/ml and diluted as needed. A calibration curve was constructed from data obtained from the HPLC analysis of serially diluted solutions (10 μl) of stock adenosine-3',5'-cyclic monophosphate (0.03 m*M*). Subsequently, the calibration curve was employed to determine the concentration of each component in the cell lysates and growth medium after analyzing aliquots (10 μl) thereof. The identity of each peak present in the experimental samples was determined by comparing the retention time of each peak with that of each N^6-cycloalkylated nucleoside and 5'-monophosphate. Further proof of the identity of each peak was obtained by the addition of an authentic sample of each compound to the cell extracts and growth medium samples before HPLC analysis, and observing the elevation in the height of specific peaks thereby indicating co-migration of each component in the experimental samples with each standard N^6-cycloalkylated nucleoside and/or N^6-cycloalkylated nucleoside-5'-monophosphate.

RESULTS AND DISCUSSION

As indicated in Fig. 1a, b, and c, one component was detected, by HPLC, in the three different cell lysate samples, derived from cells separately treated with N^6-cyclopropyl-, N^6-cyclobutyl-, and N^6-cyclopentyladenosines, respectively. Fig. 2a, b, and c indicates the presence of two components, in the three different samples of growth medium, in which the cells were treated.

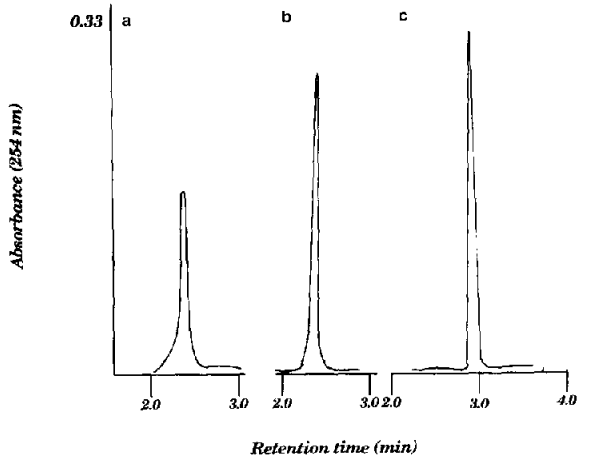

Fig. 1. Components in cell lysates. Chromatograms obtained from HPLC analysis of cell lysate samples (10 μl) of mouse hepatoma cells treated with (a) N^6-cyclopropyl-, (b) N^6-cyclobutyl-, and (c) N^6-cyclopentyladenosines (33 μg/ml), respectively. The individual peaks correspond to the resulting 5'-monophosphates. Incubation time: 24 h at 37°C. Solvent: 70% methanol–water. Flow-rate: 0.8 ml/min.

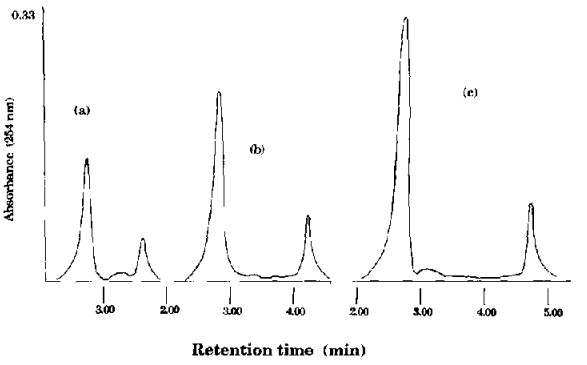

Fig. 2. Components in growth medium. Chromatograms obtained from HPLC analysis of the growth medium samples (10 μl) in which the mouse hepatoma cells were treated with (a) N^6-cyclopropyl-, (b) N^6-cyclobutyl-, and (c) N^6-cyclopentyladenosines (33 μg/ml), respectively. The first and second peaks correspond to the N^6-cycloalkylated nucleoside-5'-monophosphates and nucleosides, respectively. Solvent: 70% methanol–water. Flow-rate: 0.8 ml/min.

When each cell lysate sample was spiked with an authentic sample of the respective N^6-cycloalkylated-5'-mononucleotide, an increase was observed in the heights of the peaks, corresponding to each single component, thus indicating co-migration of each single component in the cell lysate sample with the added 5'-mononucleotide standard.

Upon the addition of an authentic sample of each N^6-cycloalkylated nucleoside and its corresponding 5'-monophosphate to an aliquot of their respective growth medium sample, and subsequent analysis by HPLC, an enhancement in the peak height of the two original components was observed. Co-migration of the two components in the three different growth medium samples, with authentic samples of their respective nucleoside and 5'-mononucleotide, suggests that they were the same as the added standards.

The retention times of each single component

TABLE I

ELUTION PATTERNS OF COMPONENTS IN CELL LYSATES AND GROWTH MEDIUM

The single component in each cell lysate sample corresponds to the respective N^6-cycloalkylated nucleoside-5′-monophosphate. Components 1 and 2 (peaks 1 and 2) in the growth medium correspond to the individual N^6-cycloalkylated nucleoside-5′-monophosphate and nucleoside, respectively. Solvent: 70% methanol–water. Flow-rate: 0.8 ml/min.

Compound	Retention time (min)		
	Cell lysate	Growth medium	
	Single component	Component 1	Component 2
N^6-Cyclopropyladenosine	2.47	2.49	3.56
N^6-Cyclobutyladenosine	2.72	2.77	4.30
N^6-Cyclopentyladenosine	3.11	2.91	4.94

in the three different cells lysate samples were essentially the same as that of the first component, peak 1, in each of the three growth medium samples (Table I) and, therefore, corresponded to each respective 5′-mononucleotide. The retention time of the second component, peak 2, in each growth medium sample corresponded to that of the particular nucleosides with which the cells were treated.

Table II reveals that there was generally an enhancement in the concentrations of the N^6-cycloalkylated 5′-mononucleotides in the cell lysates as the size of the cycloalkyl group, attached to the N^6-position of the nucleosides, increased. A similar pattern was noted for the concentrations of the two components in the growth medium. This indicates that the N^6-cycloalkylated nucleosides were either suitable substrates for adenosine kinase or their corresponding 5′-monophosphates were poor substrates for adenosine phosphorylase. Bulky alkyl groups, attached to nucleosides, are known to affect the activities of these and other enzymes [6].

The presence of one specific 5′-mononucleotide in the different cell lysate samples indicated that the N^6-cycloalkylated nucleoside was con-

TABLE II

CONCENTRATIONS OF COMPONENTS IN CELL LYSATES AND GROWTH MEDIUM

The single component in the cell lysates and the first component (peak 1) in the growth medium correspond to the respective N^6-cycloalkylated nucleoside-5′-monophosphate. The second component (peak 2) in the growth medium samples corresponds to the respective N^6-cycloalkylated nucleosides. Each value represents the mean for the analysis of three cell lysate and growth medium samples (10 μl each). Solvent: 70% methanol–water. Flow-rate 0.8 ml/min.

Compound	Concentration (μg/10 μl)					
	Cell lysate		Growth medium			
	Single component		Component 1		Component 2	
	24 h	48 h	24 h	48 h	24 h	48 h
N^6-Cyclopropyladenosine	0.40	0.07	1.26	0.96	0.80	0.74
N^6-Cyclobutyladenosine	0.57	0.25	4.15	3.65	2.04	1.72
N^6-Cyclopentyladenosine	0.79	0.52	4.36	4.20	2.21	2.03

verted to its corresponding 5′-monophosphate which was cytotoxic to the cells, causing some of them to lyse and thereby releasing the individual 5′-mononucleotide into the growth medium. The lysing of mouse hepatoma cells, by cycloalkylated nucleosides, has been observed previously [3]. It is generally acknowledged that alkylated nucleosides are only cytotoxic to various types of mammalian cells to the extent that they are converted to their corresponding 5′-monophosphates [2].

A comparison of the concentrations of the N^6-cycloalkylated 5′-mononucleotides in the cell lysates and growth medium at 24 and 48 h (Table II) suggests that considerable lysing of the cells occurred between the two incubation periods. As a result, a greater amount of the N^6-cycloalkylated 5′-monophosphates was observed in the growth medium than in the cell lysates samples. On the other hand, the concentrations of the 5′-mononucleotides as well as the nucleosides in the growth medium were less at 48 h than they were at 24 h. The decrease in concentration of the individual 5′-monophosphates in the growth medium samples may be attributed to hydrolysis of the 5′-monophosphates to the nucleosides by 5′-nucleotidase, while the diminution in the concentration of the nucleosides may be attributed to partial hydrolysis to the free bases by purine nucleoside phosphorylase [2]. Nevertheless, the relative amounts of each nucleoside and its corresponding 5′-monophosphate, in the growth medium samples, did not vary considerably from 24 to 48 h.

The detection and identification of the specific 5′-mononucleotides in the cell lysate samples and both the individual nucleosides and corresponding 5′-mononucleotides in the growth medium samples suggest that the conversion of the N^6-cycloalkylated nucleosides to the corresponding 5′-monophosphates within the mouse hepatoma cells may be the mechanism by which the N^6-cycloalkylated nucleosides exert their cytotoxic effects.

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REFERENCES

1 R. W. Trewyn and S. J. Kerr, *Biochem. Pharmacol.*, 28 (1979) 607.
2 A. Y. Divekar, M. H. Fleysher, H. K. Slocum, L. M. Kenny and M. T. Hakala, *Cancer Res.*, 32 (1972) 2530.
3 R. Thedford, E. O. Leyimu, D. L. Thornton and R. Mehta, *Exp. Cell Biol.*, 57 (1989) 53.
4 D. Perrett, in C. K. Lim (Editor), *HPLC of Small Molecules*, IRL Press, Washington, DC, 1986, p. 3.
5 M. P. Schweizer, R. Thedford and J. Slama, *Biochem. Biophys. Acta*, 232 (1971) 217.
6 B. M. Chassy and R. J. Suhadolnik, *J. Biol. Chem.*, 242 (1967) 3655.