ELSEVIER

Journal of Chromatography B, 688 (1997) 359–363

JOURNAL OF CHROMATOGRAPHY B

Short communication

Hydrophobic chromatography of the HL-60 cellular fraction co-binding with hexamethylene bisacetamide

Dongxu Xie[a,*], Shuben Li[b], Liqin Ren[a]

[a]*Administration Department of Lanzhou General Hospital of Lanzhou Command PLA, Lanzhou 730050, China*
[b]*State Key Laboratory for Oxo Synthesis and Selective Oxidation, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, China*

Received 1 April 1996; revised 18 June 1996; accepted 8 July 1996

Abstract

Methods of separating N-acetyl-1,6-diaminohexane (NADAH) and its immobilization to diol-silica have been developed. Hexamethylene bisacetamide (HMBA) and its metabolite NADAH are used as inducers of leukemia cell differentiation. The inducing mechanism of HMBA is still not clear. Experiments show that HMBA and NADAH undergo relatively strong hydrophobic reactions and do not readily undergo ion-exchange with the proteins of the cytosolic fraction of HL-60 cells during immobilization of NADAH; the retention time of the proteins was longer than that of the phosphatides. These results show that the adsorption of HMBA and NADAH to proteins was higher than that to phosphatides. The expected biospecific receptor binding with HMBA has not been found.

Keywords: Hexamethylene bisacetamide; N-Acetyl-1,6-diaminohexane

1. Introduction

Cell fate during embryogenesis and cell culture, as well as tumor progression, can be altered by a heterogeneous class of compounds known as inducers. A series of derivatives of formamide and acetamide have been found to induce the terminal differentiation of a number of animal and human solid and leukemic tumor cell lines. These compounds contain an apolar region linked to one or more polar molecular groups, so their essential features are a combination of hydrophobicity and high dipole moment. It is recognised that their target is the plasma membrane. One of these compounds is hexamethylene bisacetamide (HMBA) which has been extensively studied [1–5]. Furthermore, HMBA and its metabolite N-acetyl-1,6-diaminohexane (NADAH) have been found to possess the potential to induce the terminal differentiation of HL-60 human acute progranulocytic leukemia cells [6–8]. The NADAH has an active radical $-NH_2-$. This radical can be immobilized to study the interaction between the cytosolic fraction of HL-60 cells and HMBA or NADAH. We present evidence that NADAH and HMBA may undergo interaction with proteins. These findings will contribute further to the understanding of the action mechanisms of HMBA and NADAH.

*Corresponding author.

PII S0378-4347(96)00312-X

2. Experimental

2.1. Cell culture

HL-60 human acute progranulocytic leukemia cells were maintained in RPMI1640 (GIBCO, Grand Island, NY, USA) containing penicillin (50 unit/ml), streptomycin (50 μg/ml), L-glutamine (2 μ*M*/ml) and 10% (v/v) heat-inactivated newborn calf serum. Cells in this experiment were cultured at 37°C in an atmosphere of 5% CO_2 and 95% humidity. Each culture was initiated with ~10^5 cells per ml and up to $1 \cdot 10^7$–$2 \cdot 10^7$ cells were collected by centrifugation and washed three times in normal saline.

2.2. Cell lysis

Cells (50 μl) were lysed with 0.5 ml 0.05 *M* phosphate buffer (pH 7.4) containing 1 m*M* EDTA, 1% (v/v) Triton-100, $2 \cdot 10^{-4}$ *M* PMSF by incubation for 30 min on ice. Lysed cells were centrifuged at 400 *g* for 10 min at 4°C; the supernatant was collected and centrifuged at 10 000 *g* for 10 min at 4°C. The supernatant was used as the cytosolic fraction of HL-60 cells and stored −20°C before use.

2.3. Synthesis and purification of 6-acetamido-1-aminohexane (NADAH) hydrochloride

NADAH was synthesized by partial acetylation of 1,6-diaminohexane with acetic anhydride [8]. The resulting mixture was extracted by hot anhydrous isopropyl alcohol, the extractive solution condensed by evaporation, and the crystals formed discarded. The residue was added to the neutral Al_2O_3 column (80×2 cm I.D.), using methanol–ethyl acetate (1:1, v/v) as eluant, and the second peak analysed by TLC (stained with iodine). The pure yield material was confirmed by 80 MHz ^1H NMR (^2H_2O) as NADAH.

2.4. Immobilization of NADAH

Diol-silica and aldehyde-silica were prepared using epoxy-silica. Tosyl-silica was prepared using diol-silica. Then binding of NADAH on epoxy-silica, aldehyde-silica and tosyl-silica was carried out [9,10]. Resulting silica derivatives were determined by chemical elemental analysis and FTIR.

2.5. HPLC analysis

The derivatized silica was packed in a stainless steel column (150×4.6 mm I.D.), purchased from Beijing Analytical Equipment Manufacturing. The HPLC equipment used was a HP1090 system; UV detector wavelength was 280 to 450 nm; a 20-μl sample (protein content: 2.50±0.05 mg/ml) was used for each injection. The ion-exchange effect of the NADAH column was studied using deionized water and 1 *M* NaCl pH 7.4 as eluant. The hydrophobicity of the NADAH column was determined by studying a linear gradient of 2.0 *M* $(NH_4)_2SO_4$–0.1 *M* phosphate buffer (pH 7.4) to 0.1 *M* phosphate buffer (pH 7.4). The expected compound(s) binding biospecifically with HMBA or NADAH were identified by first using 0.15 *M* NaCl–0.1 *M* phosphate buffers (PBS) (pH 7.4) and then changing to 0.2 *M* HMBA.

3. Results and discussion

3.1. Synthesis and purification of NADAH

The residue was extracted with hot isopropyl alcohol to separate the product and a major portion of the nonreacted agent. Extracted isopropyl alcohol was evaporated by rotary evaporation to enrich the product. The crystals formed during this process were identified as staple diaminohexane hydrochloride by 80 MHz NMR, but no NADAH was observed as described in Ref. [8]. The residue obtained above was eluted through the neutral Al_2O_3 column by methanol–ethyl acetate. The first peak was HMBA, the second peak was identified by ^1H NMR (^2H_2O) as NADAH which has a characteristic peak at 1.97 ppm (CH_3CO).

3.2. Immobilization of NADAH

The results of the chemical element analysis show that the tosyl-silica gel (NADAH content of the silica: 214 μ*M*/g) has the highest coupling capacity compared to epoxy-silica (reacted for six days at

room temperature) (106 μ*M*/g) and aldehyde-silica (42 μ*M*/g). The FTIR spectrum of the tosyl-silica after NADAH binding shows the carbonyl group peak absorbed at 1747.6 cm^{-1} .

3.3. Interaction between NADAH column and the cytosolic fraction of HL-60 cells

With regard to the interaction between solute molecules and ligands immobilized on a chromatography matrix, there are three types of separation method for macromolecules: ion-exchange, affinity and hydrophobic chromatography. In ion-exchange chromatography the separation depends upon the reversible adsorption of charged solute molecules to an immobilized ion-exchange group of opposite charge, and is often performed by changing pH or ionic strength (salt gradient elution). Affinity chromatography depends upon biospecific interactions between solute molecules and ligands, and often uses the free ligands as the cleavage eluant.

Hydrophobic adsorption depends upon the interaction between solute molecules and ligands immobilized on a matrix, and is accounted for by an increase in the surface tension of water arising from the structure-forming salts dissolved in it. The basis for this mechanism is that the Van der Waals attraction forces between protein and ligand increase as the ordered structure of water increases in the presence of salting-out salts. Sodium or ammonium

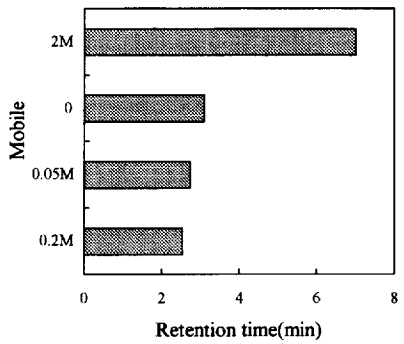

Fig. 2. Retention time of HL-60 cytosolic fraction on the NADAH column. Mobile phases (from bottom to top): (1) 0.2 *M* PB (pH 7.4); (2) 0.05 *M* PB (pH 7.4); (3) deionized water; (4) 2 *M* $(NH_4)_2SO_4$, 0.05 *M* PB.

sulphates are commonly used as the salts of "salting-out" (precipitation) or molar surface tension increment effect. It is thought that the principle of reversed-phase chromatography is similar to that of hydrophobic chromatography. However, the former has stronger hydrophobic interaction [11].

The filtration ability of the cytosolic fraction of HL-60 cells on the 5 μm silica gel with 100 Å pores is very weak (Fig. 1). As the ion concentration of the eluent increases, the retention time of the cytosolic fraction of HL-60 decreases, but overall change is negligible. The retention times of the sample are all within 5 min when 0.2 *M* PB, 0.05 *M* PB and deionized water are used as eluants (Fig. 2). This

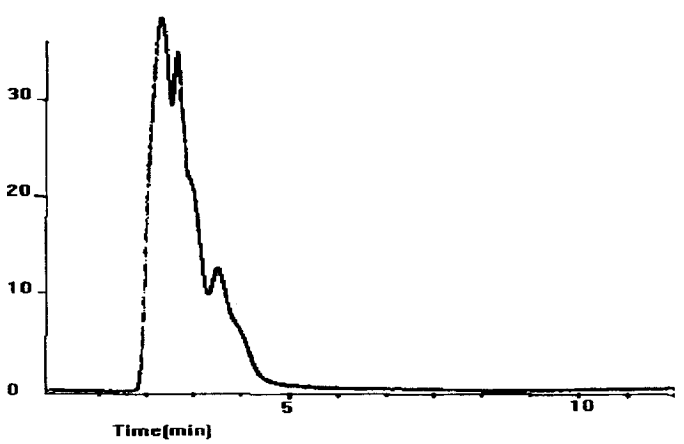

Fig. 1. HPLC spectra for the cytosolic compounds of HL-60 cell. NADAH column; flow-rate, 0.5 ml/min; mobile phase, (A) 0.01 *M* PB (pH 7.4), (B) 1 *M* NaCl, 0.1 *M* PBS (pH 7.4); gradient, in 20 min from 100% (A) to 100% (B).

result suggests that the amide radical –CONH– of HMBA and NADAH had little ion-exchange effect in the samples for HL-60 cells.

Figs. 2 and 3 show the retention times and the spectra of the cytosolic fraction of HL-60 cells on the NADAH column, using a linear gradient from mobile phase A (2.0 *M* $(NH_4)_2SO_4$–0.1 *M* PB) to mobile phase B (0.1 *M* phosphate buffer). The retention time range for the cytosolic fraction of HL-60 cell increases significantly (>7 min), when compared to Figs. 1 and 2. The starting elution for the first peak ranges from 1.99 min to 2.7 min. The HPLC spectrum of cytosolic compounds clearly consists of two groups of peaks. The hydrophobic effect of HMBA and NADAH on HL-60 cells is stronger than the ion-exchange effect. They also have differing hydrophobic adsorption to various proteins in the cytosolic fraction of HL-60. Using mobile phases of 0.1 *M* phosphate buffer and 0.2 *M* HMBA, no receptor on the HL-60 cell is found. A negative peak at about 2 min, which is formed by phosphatides following extraction by chloroform, can be seen in the above-mentioned spectra. Adsorption of HMBA and NADAH to proteins was greater than adsorption to phosphatides.

Previous observations [12,13] have shown that the electrical potential across the plasma membrane was implicated in leukemia cell commitment to differentiation by HMBA. The apolar portion of the inducer ensures preferential adsorption at the membrane–solution interface, to a certain degree. This adsorption produces a shift of the surface potential; the dependence of the surface potential upon the charge density is determined by the interplay of hydrophobic and dipolar electoral features. At the diffusion equilibrium of the inducer, the slightest difference of transmembrane potential is determined for the biological effects [12,13]. Furthermore, the present results show that HMBA and NADAH have relatively stronger hydrophobic interactions with the proteins of the cytosolic fraction of HL-60 cells than with phosphatides as it is at the diffusion equilibrium of HMBA. It is not known whether these hydrophobic interactions of HMBA and proteins are strong enough to induce the leukemia cells to differentiate or whether there are some/other proteins that will trigger differentiation by hydrophobic interaction with HMBA or NADAH.

Acknowledgments

Authors thank Professor Weile YU for the review of the manuscript, and thank Jingguo HOU for providing epoxy-silica. This work was supported by the Youth Scientist's Foundation (No. ZR-95-093) of Gan Su Province, RP of China and the State Key Laboratory for Oxo Synthesis and Selective Oxida-

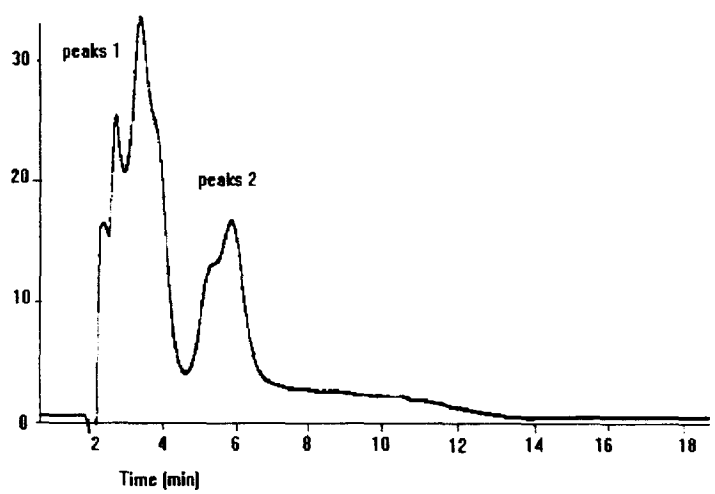

Fig. 3. Hydrophobic HPLC spectra for the cytosolic fraction of HL-60 cell. NADAH column; flow-rate, 0.5 ml/min; mobile phase, (A) 0.1 *M* PB (pH 7.4), (B) 2 *M* $(NH_4)_2SO_4$, 0.1 *M* PBS (pH 7.4); gradient, in 20 min from 100% (B) to 100% (A).

tion, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences.

References

[1] S.A. Collins, F.W. Ruscetti, R.E. Gallagher and R.C. Gallo, Proc. Natl. Acad. Sci. USA, 75 (1978) 2458.
[2] S.J. Collins, A. Bodner, R. Ting and R.C. Gallo, Cancer Res., 40 (1980) 1727.
[3] S.P. Langdon and J.A. Hickman, Cancer Res., 47 (1987) 140.
[4] R.C. Reuben, R.L. Wife, R. Breslow, R.A. Fifkind and P.A. Marks, Proc. Natl. Acad. Sci. USA, 73 (1976) 862.
[5] P.A. Marks and D.A. Rifkind, Concise Review in Clinical Experimental Hematology, Alpha Med Press, OH, 1992, p. 91.
[6] S.W. Snyder, M.J. Egorin, L.A. Geelhaar, A.W. Hamburger and P.S. Callery, Cancer Res., 48 (1988) 3613.
[7] S.W. Snyder, M.J. Egorin, E.G. Zuhowski, E. Schimpff and P.S. Callery, Cancer Commun., 2 (1990) 231.
[8] P.S. Callery, M.T. Egorin, L.A. Geelhaar and M.S. Balachandran Nayar, Cancer Res., 46 (1986) 4900.
[9] K. Nilsson and K. Mosbach, Methods Enzymol., 104 (1984) 56.
[10] P.O. Larsson, Methods in Enzymol., 104 (1984) 212.
[11] J. Porath, J. Chromatogr., 376 (1986) 331.
[12] A. Arcangeli, C.M. Marcello, D.B. Riccarda, B. Andrea, W. Enzo and O. Massime, Proc. Natl. Acad. Sci. USA, 90 (1993) 5858.
[13] A. Arcangeli, L. Ricupero and O. Massime, J. Cellular Physiol., 132 (1987) 387.