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Original Article

Facile method for determination of deoxycytidine kinase activity in biological milieus

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ABSTRACT

A new analytical method for determining deoxycytidine kinase (dCK) activity in biological milieus using luminescence is reported here. This method, based on utilizing adenosine triphosphate (ATP) as the sole phosphate donor in the kinase reaction and monitoring ATP consumption via a luciferase-based chemiluminescence reaction, is capable of detecting dCK activity without the use of specific substrates or radioisotope techniques. Comparing with the reverse-phase high-performance liquid chromatography method, this new method is suggested to be efficient and sensitive. Further, application of the proposed method for profiling dCK activity in cultured cancer cells revealed that a cervix cell line exhibited the highest dCK activity to gemcitabine metabolism.

1. Introduction

Deoxycytidine kinase (dCK) is an enzyme expressed in eukaryotic cells [1,2] that catalyzes phosphate transfer between adenosine triphosphate (ATP) and 2′-deoxynucleosides. It plays an essential role in the salvage pathway of nucleotide metabolism. Therapeutically, dCK phosphorylates anticancer nucleoside analogs such as gemcitabine (2′,2′-difluorodeoxycytidine) [3] and 2-chlorodeoxyadenosine (CdA) to their cytotoxic forms [4], which are effective chemotherapeutic agents for some solid tumors and leukemia. Clinically, dCK is reported to play a role in mediating the effectiveness of nucleoside antimetabolite therapies [5–7].

Cumulative pharmacological and pharmacogenetical data indicate that dCK activity correlates with both efficacy of gemcitabine therapy and resistance to the drug [5–10]. The dCK overexpression increased the sensitivity of colon, breast, and lung cancer cells to gemcitabine [7,11]; by contrast, dCK downregulation enhanced acquired drug resistance in pancreatic cells [6,11]. Levels of dCK protein expression in human pancreatic cancer tissues are correlated with the overall survival of the gemcitabine-treated patients [10,12]. The combined evidence suggests that dCK is a versatile biomarker in nucleoside anticancer therapy and governs the effectiveness of gemcitabine treatment.

Only a few methods have been established for detecting dCK in biological samples. Antibody-based methods such as western blotting and enzyme-linked immunosorbent assay have limited ability to detect the active form of dCK; therefore, antibodies that can recognize various dCK substrates are

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required [13,14]. Conventional assays for dCK, based on the use of radioactive substrates such as [8-3H] CdA and a series of binding and washing procedures to separate "hot" and "cold" portions to assess activity [4,14], are complicated with the use of radioisotope materials, which involves problems such as waste disposal and radiation safety. Bierau et al [15] developed a high-performance liquid chromatography (HPLC) method using nonradiolabeled CdA with inherent limitation in sensitivity because nucleosides have no specific chromophores for spectrophotometric detection. Therefore, in order to explore impacts of dCK in pharmacological and clinical studies, development of an efficient and sensitive method to determine dCK activity in biological milieus is mandatory.

ATP has been found to serve as the phosphate donor in the dCK reaction, and ATP analysis is correlated to detect dCK activity enzymatically (Fig. 1). Several highly sensitive fluorescence and luminescence methods have been developed for the discovery of kinase inhibitors in preclinical studies [14]. We accordingly developed an efficient luminescent assay for determining dCK activity in biological milieus. The assay is based on the use of anion exchange beads to purify dCK from complex biological samples and a luciferase-based ATP assay to quantitate dCK activity. With ATP serving as the sole phosphate donor to dCK substrates in the proposed assay reaction (Fig. 1), ATP consumption, monitored by a luciferase-based chemiluminescent reaction, can be utilized to determine the enzymatic activity of dCK. In the reaction, the luminescent signal generated from the conversion of beetle luciferin to oxyluciferin with the stoichiometry of one ATP molecule to one photon per turnover is inversely correlated to the dCK activity. This new method has been validated with comparison to the conventional HPLC method. Its application in profiling dCK activity in cancer cells has also been evaluated.

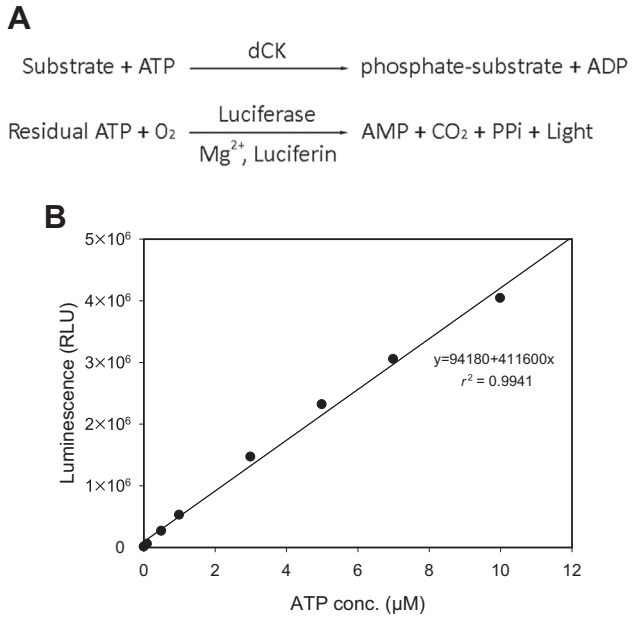

Fig. 1 – (A) Reaction scheme of the luminescence dCK assay. Given that the phosphate transfer reaction consumes one ATP and one oxygen molecule to produce one photon of light in the luciferase-based system, the light measured as RLU is correlated with the amount of residual ATP and inversely reflects dCK activity. (B) A linear relationship is observed between the luminescence signal and ATP concentration. The correlation coefficient (r^2) is 0.9941 for ATP in the concentration range of 0.1–10μM. ADP = adenosine diphosphate; ATP = adenosine triphosphate; dCK = deoxycytidine kinase; RLU = Relative light unit.

2. Methods

2.1. Materials

Deoxycytidine and deoxycytidine triphosphate (dCTP) were purchased from Sigma-Aldrich (St Louis, MO, USA). Gemcitabine (2′,2′-difluorodeoxycytidine) was obtained from Innopharmax (Taipei, Taiwan). Kinase-Glo reagent was purchased from Promega (Madison, WI, USA). Other chemicals were of analytical grades and obtained from Sigma-Aldrich. Milli-Q water prepared by a Millipore system was used in buffer preparation.

2.2. Cell lines

HeLa, HCT116, LX-1, 780-O, and MCF-7 cells were obtained from the American Type Culture Collection (10801 University Boulevard Manassas, VA 20110 USA). In brief, HeLa and MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), HCT116 and 786-O cells were cultured in RPMI-1640, and LX-1 cell was cultured in Minimum Essential Medium. All media were supplemented with 10% Fetal Bovine Serum (FBS); and cells were maintained at 37°C in 5% CO_2 and routinely passaged every 2–3 days in a 75T flask.

2.3. Protein extraction and quantitation

Cell pellets suspended in extraction buffer (PRO-PREPTM Protein Extraction solution; iNtRON, Kyunggi-do, Korea) were sonicated twice on ice for 10 seconds each. After sonication, extracts were incubated at −20°C for 10 minutes and centrifuged at 13,628*g* at 4°C for 10 minutes. Supernatants containing the cellular proteins were collected and maintained at −20°C prior to analysis.

Protein concentrations of the cell extracts were determined by a Pierce 660 nm Protein Assay (Thermo, 81 Wyman Street, Waltham, Massachusetts 02454, USA) using bovine serum albumin as the protein standard. The calibration curve was constructed by plotting the optical densities obtained at 660 nm versus the individual known bovine serum albumin concentration ranging from 0.1 mg/mL to 2.0 mg/mL.

2.4. Developed luminescent assay

To distinguish dCK from other kinases that may interfere with the assay, a quick purification procedure adapted by Quan et al [16] was employed. In brief, quantitated cell extracts containing dCK were incubated with 0.5 mL HiTrap Q