Identification of a Previously Unknown Compound as 2'-Deoxyctydine Found in the Plasma of Breast Cancer Patients under Combined Chemotherapy

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In an attempt to find an end-point for cancer chemotherapy, this study was designed to measure the adenine compounds in the plasma of breast cancer patients using HPLC with a selective reagent for adenine bases. The patients were treated by chemotherapy using cyclophosphamide, methotrexate and 5-fluorouracil. Blood was collected in tubes containing EDTA, the plasma separated by centrifugation and analysed by HPLC. An early peak due to the fluorescent derivative of an unknown compound reacted with bromoacetalddehyde and its concentration appeared proportional to the chemotherapeutic courses of treatment. The compound in its native state without fluorescent derivatization was efficiently purified by using columns of DEAE- and CM-Sephadex. Its UV spectrum revealed maxima at 271, 280 and 272 nm in solutions of pH 7, pH 3 and pH 12, respectively. The electrophoretograms showed that it was neutral, positively and negatively charged at pH 7, pH 3 and pH 12, respectively. Thin-layer chromatograms showed that it had the same RF as 2'-deoxyctydine (dCyd) which was confirmed by a positive reaction for deoxyribose. It was concluded that bromoacetalddehyde formed a weakly fluorescent product with dCyd which gave rise to the early peak in the anion exchange chromatograms. From the calculation of the recovery obtained by the purification process, the cancer patients undertaking more than 12 courses had a dCyd level of approximately 20 μM while the corresponding figure in normal volunteers was less than 1 μM. These results may be useful in assessing the status of the cancer patients.

Keywords 2'-deoxyctydine; breast cancer; chemotherapy; adenine; HPLC

In spite of the prominent role played by chemotherapy in the overall management of different types of cancer, early detection of a response or determination of the end-point of treatment is still empirical. There has never been any evidence that any special compound is produced by patients when such drugs are effective. Many of the available anticancer agents either act directly on DNA as alkylating agents or combine to inhibit DNA synthesis, such as methotrexate and 5-fluorouracil. Therefore, it is to be expected that drugs like this well give rise to changes in nucleosides levels. The excrated or substituted nucleosides may be detected in the plasma and urine of treated patients.

Many authors have reported that increasing amounts of purine and pyrimidine nucleosides are excreted in the urine of tumor-bearing animals and also in humans. For example, a higher level of methylated purine has been demonstrated in urine of patients with acute Leukemia and Kaufman has reported a resistance to 5-fluorouracil associated with increased cytidine 5'-triphosphate levels in V-79 Chinese Hamster cells.

Yoshioka et al. have developed a sensitive fluorimetric method for determining adenine-containing compounds in plasma and urine of patients and volunteers. It is based on the HPLC separation of fluorophores derived from the interaction of adenine-containing compounds with a new fluorescence reagent, bromoacetalddehyde. This method can be made specific for adenine derivatives using a selective detection and separation system.

The present work was originally aimed at applying this method to investigate whether any adenine derivative might be used as a treatment parameter for cancer chemotherapy, since the metabolic pool of adenine derivatives is assumed to be potentially large compared with those of the other nucleotide base derivatives. In fact, an early peak due to an unknown compound in the anion exchange chromatograms appeared proportional to the number of chemotherapeutic courses. This paper reports efforts to identify the unknown compound using various analytical methods.

MATERIALS AND METHODS

Samples Blood was collected from 60 Egyptian breast cancer patients who had received radical mastectomy and radiotherapy and were undergoing chemotherapy; blood from 20 normal subjects was corrected as controls. The breast cancers were mostly late-occurring. The 20 control subjects were healthy staff and nurses of the National Cancer Institute, Cairo. No other treatment was given for at least 30 d before initiation of chemotherapy with cyclophosphamide (endoxan), methotrexate and 5-fluorouracil (CMF). The three drugs were administered successively by the i.v. route slowly over a period of 15 min. The course was repeated every 21 d. If leukopenia was produced, the course was delayed until the next week. The data are presented in Fig. 2 as groups of patients taking a maximum of 5, 10, 15 or more than 16 courses, respectively. Each group represented around 15 patients. Details will be described elsewhere. The blood was collected over EDTA (2 mg/ml) and the plasma separated by centrifugation at 3500 × g at 4 °C for 10 min. The plasma samples were immediately stored at −80 °C until analysis of the adenine nucleoside and nucleotides was per-
formed.

Sample Preparation for HPLC\(^7\) One hundred micro-
liters of the sample was added to 25 \(\mu\)l 2 M perchloric
acid and the mixture centrifuged at 15000 \(\times g\) for 30 min.
Fifty microliters of the supernatant was added to 10 \(\mu\)l
2 M potassium hydroxide and the mixture centrifuged at
3500 \(\times g\) for 5 min. To 50 \(\mu\)l of the supernatant an equal
volume of 0.2 M phosphate buffer (pH 7.0) was added. To
50 \(\mu\)l of this mixture 5 \(\mu\)l 1.9 M bромoacetalddehyde was
added. A reaction vial with a screw cap containing this
mixture was heated at 80 °C for 15 min then stored at 4 °C
until HPLC analysis. The standard samples were prepared
by dissolving several different concentrations of adenine
compounds in 0.2 M phosphate buffer (pH 7.0). An aliquot of
50 \(\mu\)l of each concentration was reacted in the same
manner.

HPLC\(^7\) A column (10 cm \(\times\) 4.6 mm) of Hitachi gel No.
3012-N (porous polystyrene polymer beads for anion
exchange, mean diameter of 7 \(\mu\)m, Hitachi, Tokyo, Japan)
was maintained at 45 °C. The eluent consisted of 0.025 M
citric acid–0.05 M disodium hydrogen phosphate–0.4 M
sodium chloride buffer (pH 5.0) and methanol (1: 1, v/v).
The flow-rate of the eluent was set at 0.2 ml/min using a
Twincle pump (Jasco, Tokyo). The column inlet pressure
was 10 kg/cm\(^2\). The column was connected to a
fluorescence detector FLD-1 (Shimadzu, Kyoto, Japan)
equipped with a low-pressure mercury lamp of maximum
energy at 253.7 nm, a Shimadzu EX-primary filter that
transmitted radiation in the range 250–400 nm, a quartz
flow-through cell of 10 \(\mu\)l capacity and an EM 3 secondary
filter that transmitted radiation of wavelengths above
400 nm.

Purification Process An aliquot (0.5 ml) of the
deproteinized supernatant from a patient blood sample
containing a large early peak, without performing
the fluorescent reaction, was transferred to a column
(5 cm \(\times\) 5 mm) of DEAE-Sephadex (free form). The
column was washed with 2.5 ml water. The eluate was
divided into 10 fractions of 300 \(\mu\)l each. The UV-absorbing
fractions (1–3) were pooled and transferred to a column
(5 cm \(\times\) 5 mm) of CM-Sephadex (H-form) and 4 ml 1 M
HCl was used as eluent. The eluate was divided into 10
fractions of 500 \(\mu\)l each. The UV-absorbing fractions
(4–6) were pooled and used for identification of the
unknown compound by means of various analytical
methods.

Measurement of UV Absorption Spectra This was
performed using a double-beam spectrophotometer
UVIDECC-610 (Jasco, Tokyo). The pooled solution (0.5 ml)
was diluted 5 times with the following buffers, 0.01 M citrate
buffer, pH 3, 0.01 M phosphate buffer, pH 7 and 0.01 M
borate solution, pH 12.

Electrophoresis This was carried out using an
electrophoresis apparatus Pulser HP-10 (Toyko Co.,
Tokyo) with 0.01 M citrate buffer, pH 3, 0.01 M phosphate
buffer, pH 7 and 0.01 M borate solution, pH 12. The
electrophoresis was for a distance of 6 cm at 1 mA/cm
for 5 min. The pooled solution was applied to the middle
of a cellulose acetate membrane, Separax (6 \(\times\) 10 cm)
(Jookoo Co., Tokyo). Glucose, \(\varepsilon\)-(2,4-dinitrophenyl)-\(\varepsilon\)-lysine
(DNP-Lys) and bromocresol green (BCG) were used
as standards. Glucose (Glu) was detected using 0.1 N
AgNO\(_3\) and NH\(_4\)OH (1/1, v/v) with drying at 100 °C for
5 min, when a brown colour appeared for a positive glucose
spot. DNP-Lys and BCG were visible.

TLC An aliquot of the pooled solution was spotted
on silicagel TLC plates containing a fluorescent mixture
(Merck Co.), and developed with 4 different solvent
systems. The cytosine derivatives were used as standards
as shown in Fig. 5. Deoxyribose was detected using the
diphenylamine method reported by Burton.\(^8\) The other
nucleosides were also compared with the unknown
compound by TLC.

RESULTS

Typical chromatograms of plasma samples from the
controls and breast cancer patients are shown in Fig. 1.
In general there are only two peaks, the early peak and
the peak of ATP, although adenosine, adenosine
5' monophosphate (AMP), cAMP, adenosine 5'-diphos-
phate (ADP) and ATP in this order were well separated
on this anion exchange column. The early peak had a
retention time corresponding to neutral adenine deriva-
tives such as adenine and adenosine. The same fluores-
cent derivatized samples were analyzed by a type of reverse-
phase chromatography using a column of porous poly-
styrene polymer beads, Hitachi gel No. 3012, as described
in our previous paper.\(^8\) The retention time of the unknown
peak was not overlapped by adenine and adenosine.
The early peak indicated a significant, high level of the
unknown compound in the plasma of breast cancer
patients related to the number of CMF courses, as shown
in Fig. 2.

The unknown compound in the early peak was purified
from the blood samples without reaction with bromoace-
taldehyde by using the simple process described in the
material and methods. The purified solution exhibited a
single spot based on UV absorption by TLC and elec-
trophoresis. The recovery of the extraction process was
calculated by HPLC and UV absorbance at 260 nm as

![Fig. 1. Typical Chromatograms of Blood Samples from Normal Volunteers (a) and Breast Cancer Patients (b) RFI, relative fluorescence intensity.](image-url)
97±4% (n = 10).

The compound was identified by a series of analytical techniques. The UV spectrum revealed maxima at 271, 280 and 272 nm in solutions of pH 7.0, 3.0 and 12, respectively as shown in Fig. 3. The spectra almost overlapped with those of cytosine (Cyt) base derivatives. In particular, the spectrum of 2'-deoxyctydine (dCyd) completely overlapped that of the purified sample.

The compound exhibited a neutral, positive and negative charge at pH 7.0, 3.0 and 12, respectively during electrophoresis as in Fig. 4. From the electrophoretogram at pH 12, the compound appeared to be dCyd.

The compound was separated and compared with Cyto-containing compounds by TLC as in Fig. 5. Figure 5a shows samples for two different patients (as examples) with the same Rf value and the compound is not an adenosine or adenine derivative. Figure 5b and 5c compare the behaviour of this compound with Cyto-containing compounds in acidic and alkaline media by TLC, respectively. Cyd and dCyd are not completely separated under the conditions described earlier. Figure 5d shows the separation of dCyd from Cyd in the form of the borate complex. The compound has the same Rf value as dCyd appearing in Fig. 5d. From the positive reaction with the diphenylamine for deoxyribose, the compound was identified as dCyd.

The selective increase in dCyd exhibited by cancer patients undergoing chemotherapy has been clearly established. The dCyd level, based on the UV absorption at 260 nm of purified samples from patients undertaking more than 12 CMF courses, was around 20 μM compared with less than 1 μM found in normal volunteers.

DISCUSSION

Cancer causes many changes in the host by increasing the rate of metabolism and impairing the functions of almost all the body organs. Cancer of the breast is one of the main types of cancer in females. In Egypt, breast cancer is detected at an advanced stage. In these patients, surgery usually followed by radiotherapy and chemotherapy is a common method of treatment and such patients were selected for the study described here.

A disturbance in the balance of nucleic acids is expected in these patients after treatment with chemotherapy or radiotherapy. Detection of a compound that could be used as an indicator of successful treatment would be very valuable clinically. During the analysis described earlier, an unknown compound has been found which can be efficiently and easily purified because of its higher concentration in patient samples. This unknown compound was identified as dCyd.

In other studies, many authors have reported an in-
increased excretion of methylated purine as pseudouridine in the urine of some tumor-bearing mammals, including cancer patients, but little is known about the increase of Cyt and dCyd in urine. A dCyd increase has been reported following whole body irradiation in humans and rats, treatment with radiomimetic alkylating agents (methylmethane sulfonate) in rats and in rats transplanted with Yoshida ascites sarcoma. The mechanism for the increase in dCyd has been reported by Shimizu and Fujimura as being due to an altered pyrimidine metabolism in not only Yoshida ascites sarcoma cells but also in the host organs, particularly the liver. Using blood from a cancer patients, nucleic acid metabolites were analysed by HPLC. However, no specific metabolites have been detected.

In the present work it was found that dCyd levels increased in the plasma of breast cancer patients treated with CMF and this has been found generally to be related to the number of treatment courses. Individually, patients with a bad prognosis exhibited this increase in dCyd, but those with a good prognosis had normal levels. More details will be described in a future paper. This finding involving dCyd may be due to the fact that the breast cancers in this paper were very large in size and at an advanced stage.

Bromocetoildehyde reacts with Cyt base, although the fluorescent intensity of the product is weak compared with adenine. The high concentration of dCyd produced a large early peak during HPLC. There are many well-known antimetabolite analogs of Cyt used in chemotherapy such as ara-C. The present finding suggests that dCyd may play a potential role in tumor cell metabolism.

In conclusion, we have found that dCyd levels in body fluids can be used as a possible parameter for monitoring the therapeutic effects of cancer chemotherapy. Further work is in progress applying this finding to other types of human tumors.

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