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Chemical and biological stability of anticancer drugs used in a human tumor clonogenic assay

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Summary. *Human tumor clonogenic assays (HTCA) are being used to evaluate the chemosensitivity of human cancers to both standard and experimental anticancer drugs as well as to predict clinical tumor response and resistance to these agents. To enable us to design and accurately interpret drug assay data we quantitated the chemical and biological stability characteristics of various cell cycle-specific and cell cyle-nonspecific drugs commonly used in our laboratory for chemosensitivity testing in the HTCA. Aliquots of the culture media were obtained immediately after drug addition and after 6 and 24 h and 2, 4, and 10 days of incubation, and then frozen in liquid nitrogen for later chemical (HPLC or RIA) and biological (HTCA) assay. Adriamycin, actinomycin D, bisantrene , bleomycin, and vinblastine retained 80–100% of their biological activity against human tumor colony-forming units even after 10 days of incubation in culture media. In contrast, etoposide lost 60% of its in vitro antitumor activity over the same period. There was no loss in the chemical concentration of actinomycin D. bisantrene, bleomycin, or vinblastine in the culture media, but etoposide concentrations as measured by HPLC decreased to zero over 10 days. We conclude that the HTCA can be used as a simple test for biological stability of new investigational agents prior to the development of adequate chemical assay methodology, and that it can help clarify whether a drug's in vitro antitumor activity is due to the parent compound or to a degradation product.*

Introduction

The human tumor clonogenic assay (HTCA) has proven useful in the evaluation of the chemosensitivity of human cancers to both standard and experimental anticancer drugs [3, 16, 17] and also in the prediction of clinical tumor response and resistance to these agents [2, 3, 16–18, 21]. In this assay fresh human tumor cells are exposed in vitro to anticancer drugs for either 1 h prior to plating or continuously in the culture plate [1, 4, 16]. Accurate interpretation of the assay results necessitates determination of the chemical and biological stability of these anticancer drugs for 1 h and during continuous (i.e., up to 10 days) exposures. Little information is available concerning the stability of these agents when placed in cell culture media. In this study, we have quantitated the chemical and biological stability of various cell cyle-specific [6, 19, 20] (e.g., bleomycin, etosposide, and vinblastine) and cell cycle-nonspecific [6, 19, 20] (e.g., adriamycin, actinomycin D, and bisantrene) drugs commonly used in our laboratory for chemosensitivity testing in the HTCA. The results of these and future drug stability studies should prove useful in the design and interpretation of drug assay data.

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Materials and methods

Drugs. Stock solutions of IV formula adriamycin (Adria Laboratories, Columbus, OH), actinomycin D (Merck, Sharp & Dohme, West Point, PA), bisantrene (American Cyanamid, Pearl River, NY), vinblastine (Eli Lilly, Indianapolis, IN), bleomycin (Bristol Laboratories, Syracuse, NY), and etoposide (VP-16-213) (Bristol Laboratories, Syracuse, NY) were used.

Drug stability studies. Anticancer drugs were added to enriched CRML 1066 medium plus 15% horse serum, which is used for the upper layer in the HTCA and incubated at 37° C for up to 10 days. The drugs were also added to standard CRML 1066 without horse serum. Aliquots of the culture media were obtained immediately after drug addition and after 6 and 24 h and 2, 4, and 10 days of incubation, and then frozen in liquid nitrogen (−120° C) for later chemical and biological assay in the HTCA.

To determine the effect of 0.3% agar, which is incorporated into the upper layer of the HTCA, adriamycin, and bisantrene were added to standard agar plates and incubated for 1 and 4 days at 37° C. Thereafter the diluted agar containing medium was filtered with no. 25 gauze. The biological stability of adriamycin and bisantrene were then evaluated by means of the HTCA. The chemical stability of bisantrene was determined by applying an HPLC assay [13, 14].

All seven drugs evaluated in this study caused an exponential reduction in tumor colony-forming units (TCFUs) with increasing drug dose. To allow accurate chemical and biological detection for up to 10 days of incubation, drug concentrations tested were those reproducibly associated with an approximately 70–80% inhibition of TCFUs after a 1-h exposure in the HTCA. These concentrations were 0.1 μg/ml for actinomycin, 1.0 μg/ml for bleomycin, adriamycin, bisantrene, and vinblastine, and 10.0 μg/ml for etoposide. At these drug concentrations the coefficient of variation in the inhibition of TCFUs from the two human cell lines used in these studies was less than 10%.

Human tumor clonogenic assay. The clonogenic assay has been described previously [9, 16, 17]. In the current study, cell lines rather than fresh tumors were utilized. A single cell suspension from each human tumor cell line was incubated with the freshly prepared drugs for 1 h to serve as a control. The HEC-1A endometrial carcinoma cell line was used to study adriamycin, bisantrene, bleomycin, etoposide, and vinblastine stability. Actinomycin D stability was evaluated using the HA-1 melanoma cell line. The drug concentrations tested were as described above. Simultaneously, the thawed aliquots of drug incubated in culture media were exposed to the tumor cell suspensions for 1 h. Thereafter the cells were washed twice and prepared for culture. Plates were incubated at 37° C in a humidified atmosphere containing 6% CO_2 for 10–14 days. Plates with at least 30 colonies 60 μm in diameter were counted with an automated image analysis system (Bausch and Lomb Omicon FAS II).

The survival of TCFUs after 1-h exposure to freshly prepared drug was compared with survival of TCFUs after 1-h exposure to drug pre-exposed at 37° C for different time intervals. The difference in survival was expressed as percent loss of activitity.

Human tumor cell lines. The HEC-1A endometrial carcinoma cell line was kindly provided by Dr J. Fogh (Memorial Sloan Kettering Cancer Center, New York). The cells were cultured in McCoy's 5A with 10% FCS and split every 8–10 days with Tyrode's solution (Gibco, Gand Island, NY). Forty thousand cells were seeded per plate.

The HL-A melanoma cell line was developed in our laboratory by Dr Christian Ludwig [11]. The cells were cultured in RPMI 1640 supplemented with 10% FCS and subcultured every 7–10 days with trypsin-EDTA (0.25% Gibco). Sixty thousand cells were seeded per plate.

Chemical assay procedures. Bisantrene was assayed by a HPLC method developed by Peng et al. [13, 14]. Actinomycin D concentrations were determined using a radioimmunoassay developed by Brothman et al. [5]. Vinblastine was also assayed by RIA developed by Dr M. A. Root of Eli Lilly Co., Indianapolis, IN. Finally, bleomycin and etoposide were quantitated by Dr Donald Van Harken, Bristol Laboratories, Syracuse, NY, bleomycin by RIA, and etoposide by HPLC.

Statistical analysis. Two standard error confidence intervals were calculate for each drug stability data point determined in the HTCA. For comparison between chemical (i.e., HPLC or RIA) and biological drug activity (i.e., HTCA), at each time point the two standard error confidence interval surrounding the HTCA-determined drug activity defined the confluence of the data from the two assays.

Results

Biological stability studies

Figures 1–6 show the biological activity of adriamycin, actinomycin D, bisantrene, bleomycin, vinblastine, and etoposide tested for their biologic stability in the HTCA after 6 and 24 h, and 2, 4, and 10 days of preincubation in enriched CRML 1066 at 37° C. For each of these drugs there was no difference in their in vitro activity against TCFUs whether preincubations were in plain media or in enriched CRML 1066 plus 15% horse serum.

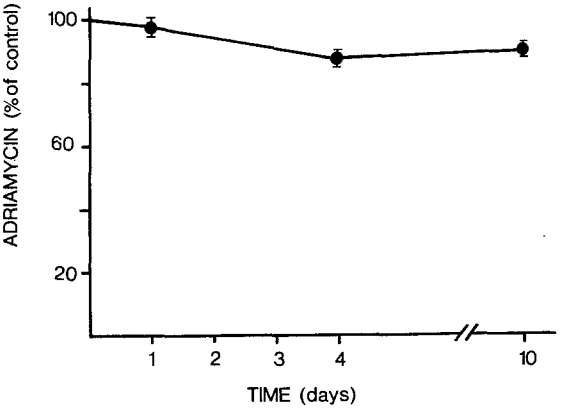

Fig. 1. Stability of adriamycin after incubation in enriched CRML 1066 (plus 15% horse serum) for 1, 4, and 10 days at 37° C. ●——●, inhibition of HEC-1A endometrial carcinoma cell line tumor colony-forming units (TCFUs) as percent of control. Control (= 100%) is activity of 1 μg freshly prepared adriamycin/ml

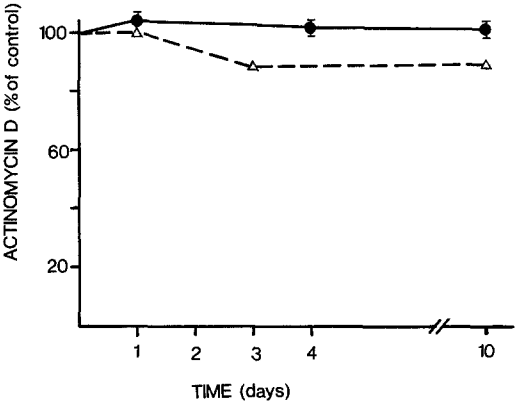

Fig. 2. Stability of actinomycin D after incubation in enriched CRML 1066 (plus 15% horse serum) for 1, 3, 4, and 10 days at 37° C. ●——●, inhibition of HA-L melanoma cell line TCFUs as percent of control; △– – –△, concentration measured by RIA and expressed as percent of control. Control (= 100%) is activity of 0.1 μg freshly prepared actinomycin D/ml

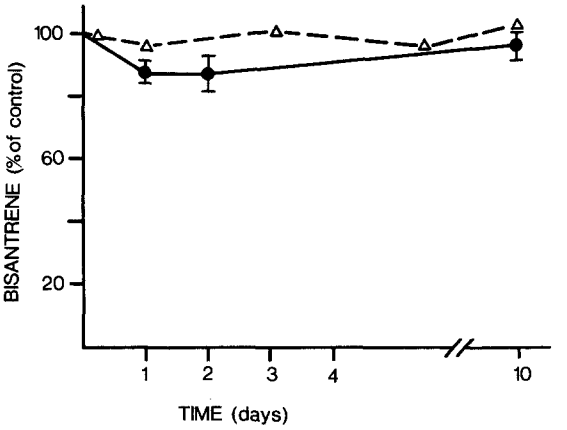

Fig. 3. Stability of bisantrene after incubation in enriched CRML 1066 (plus 15% horse serum) for 1, 2, 3, 6, and 10 days at 37° C. ●——●, inhibition of HEC-1A endometrial carcinoma cell line TCFUs as percent of control. △– – –△, concentrations measured by RIA and expressed as percent of control. Control (= 100%) is activity of 1 μg freshly prepared bisantrene/ml

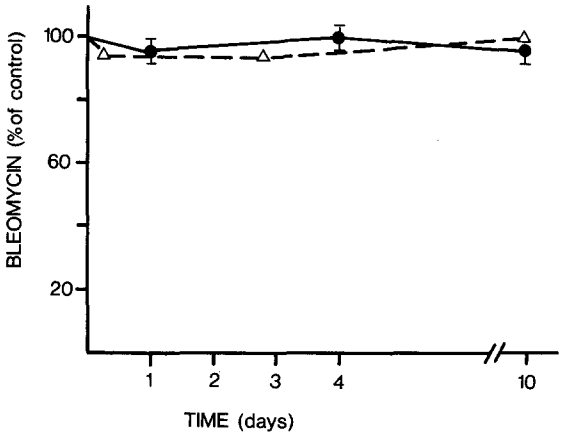

Fig. 4. Stability of bleomycin after incubation in enriched CRML 1066 (plus 15% horse serum) for 1, 3, 4, and 10 days at 37° C. ●——●, inhibition of HEC-1A endometrial carcinoma cell line TCFUs as percent of control; △– – –△, concentration measured by RIA and expressed as percent of control. Control (= 100%) is activity of 1 μg freshly prepared bleomycin/ml

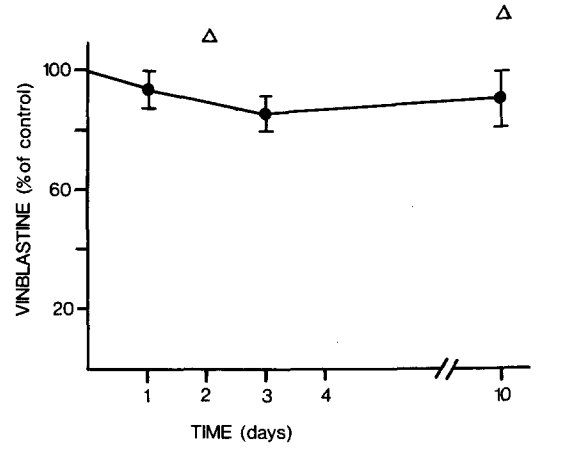

Fig. 5. Stability of vinblastine after incubation in enriched CRML 1066 (plus 15% horse serum) for 1, 2, 3, and 10 days at 37° C. ●——●, inhibition of HEC-1A endometrial carcinoma cell line TCFUs as percent of control; △ concentration measured by RIA and expressed as percent of control. Control (= 100%) is activity of 1 μg freshly prepared vinblastine/ml

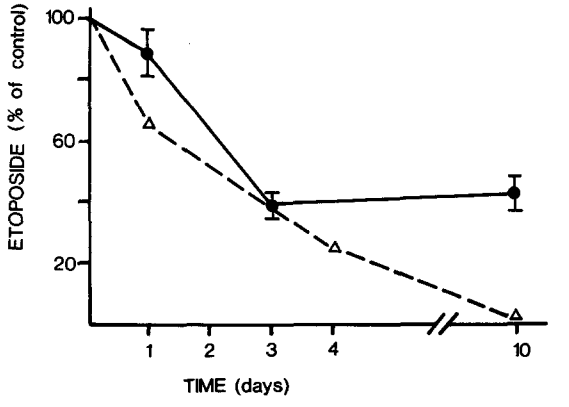

Fig. 6. Stability of etoposide after incubation in enriched CRML 1066 (plus 15% horse serum) for 1, 3, 4, and 10 days at 37° C. ●——●, inhibition of HEC-1A endometrial carcinoma cell line TCFUs as percent of control; △– – –△, concentration measured by RIA and expressed as percent of control. Control (100%) is activity of 10 μg freshly prepared etoposide/ml

Adriamycin, actinomycin D, bisantrene, bleomycin, and vinblastine retained 80–100% of their biological activity over a period of up to 10 days (Figs. 1–5). In contrast, etoposide lost 60% of its in vitro activity against TCFUs over a 10-day period (Fig. 6). There was no significant loss in adriamycin's or bisantrene's anticancer activity following incubation in CRML 1066 plus 0.3% agar for 24 h or 4 days.

Chemical stability

Figures 2–6 show the concentration-versus-time curves for actinomycin D, bisantrene, bleomycin, vinblastine, and etoposide after 1, 2, 4, and 10 days of incubation in enriched CRML 1066. Note that there was no loss in the chemical concentration of actinomycin D, bisantrene, bleomycin. Vinblastine concentrations showed some flucuations but no significant decreases in concentrations; however, etoposide's concentrations as measured by an HPLC assay disappeared completely over the 10-day incubation period. A possible metabolite appeared as an unidentified HPLC peak by 24 h and increased over time. Because of the well described problems of instability in the presence of ultraviolet light [8], extraction from biological fluids [15], and sorbent removal in vitro [22], we were unable at this time to determine accurately the chemical stability of adriamycin in the HTCA culture media.

The concentrations of actinomycin D, bisantrene, and bleomycin in the culture media were found to be similar whether measured by chemical or biological assay. For etoposide, concentrations measured by HPLC were significantly lower than those measured by biological assay in the HTCA (Fig. 6).

Discussion

The human tumor clonogenic assay has proven useful in the evaluation of the chemosensitivity of various human cancers to both standard and experimental drugs [3, 16, 17]. In addition, the HTCA has a reported 60%–70% accuracy rate in the prediction of clinical response and a greater than 85% accuracy rate in the prediction of clinical resistance to single-agent chemotherapy [2, 3, 16, 17, 19, 21]. Although chemical stability data exist for the majority of the standard anticancer drugs used in this assay, most of the standard and experimental agents have not been evaluated for chemical or biological stability under actual cell culture conditions in the HTCA. Such stability data are needed for a full understanding of the degree of antitumor activity shown by any of these agents against human tumors tested in the HTCA.

In the present study actinomycin D, bisantrene, bleomycin, and vinblastine showed stability of both chemical and biological activity while incubated in enriched CMRL 1066 at 37° C for up to 10 days. Although technical difficulties with the HPLC assay used for adriamycin [8, 15, 22] precluded the accurate quantitation of its chemical stability in assay medium, our clonogenic assay data showed adriamycin to retain greater than 80% of its biological activity over the 10-day incubation period. Of the drugs we studied only etoposide, an epipodophyllotoxin derivative [7, 10] active in the treatment of both testicular cancer and small cell cancer of the lung, showed a significant loss of both chemical and biological activity over the 10-day trial period. It is of interest that the HPLC analysis of etoposide stability revealed complete disappearance of the parent compound whithin 10 days of incubation. During this

time period a potential metabolite of etoposide was detected by HPLC. The fact that etoposide's biological activity was 40% at 10 days suggests that the persistence of this activity was related to the appearance of an etoposide metabolite. Earlier reports have documented limited chemical stability for etoposide [10], but little is known concerning the biological activity of its degradation products.

Previous data support our findings of a high degree of in vitro chemical stability for actinomycin D, bisantrene, bleomycin, and vinblastine [5, 12–14; M. Root, personal communication, 1982). It was to be expected, although not shown previously, that the biological stabilities of these agents parallel their chemical stabilities. Thus, our data suggest that the HTCA can be used as a simple test for biological stability of new investigational agents prior to the development of adequate chemical assay methodology. This biological stability may or may not reflect in vitro chemical stability. Obviously, drugs that undergo spontaneous chemical catabolism to biologically inactive compounds will be accurately evaluated for their stability by testing in the HTCA. In contrast, those agents which are chemically unstable in vitro and catabolized to biologically active compounds would be inacccurately assessed in the HTCA.

Preincubation of an anticancer drug in culture medium for up to 10 days has proven a useful test of the bioavailability of both standard and experimental drugs to be used in the HTCA. For those drugs that undergo rapid loss of biological activity following incubation in the culture medium, in vitro dose adjustments must be made to guarantee an adequate concentration × time product for the drug when it is exposed to tumor cells. For example, if it is planned to maintain a drug concentration of 1 μg/ml throughout the 10-day continuous exposure period and it is determined that a drug like etoposide maintains only 40% of its biological activity at 10 days, etoposide concentrations must be refurbished daily to maintain the concentration × time product at the desired level.

We must caution that the bioavailability studies for standard and experimental drugs used in the HTCA do not take into consideration either metabolism to biologically active compounds or uptake into the intracellular space with resultant decrease in extracellular concentration. Although for most drugs the biological and chemical stability results are likely to be complementary, divergent results between these two assay methods would provide the basis for a search for both active and inactive metabolites.

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