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Evaluation of anticancer drug schedule dependency using an in vitro human tumor clonogenic assay

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Summary. *A human tumor clonogenic assay (HTCA) has been used to evaluate standard and experimental anticancer drugs with respect to their inhibition of clonogenicity of both fresh human cancers and human tumor cell lines. By comparing the inhibitory effect on tumor colony-forming unit (TCFU) growth of 1-h and continuous drug exposures in the HTCA we were able to identify and separate schedule-dependent (e.g., bleomycin, vinblastine, and etoposide) and schedule-independent drugs (e.g., actinomycin D, adriamycin, bisantrene, and cis-platinum). Vinblastine, bleomycin, and etoposide, which are known to have 'cell cycle-specific' characteristics, caused exponential reduction in tumor colony formation when given by continuous exposure, whereas when given with a short exposure each of these drugs caused plateau-type dose-response curves. For comparison of the relative efficacy of the two dosing schedules, a ratio (1-h versus continuous exposures) was calculated of the drug concentrations which reduced growth of TCFU to 50% of the control values (ID_{50}) for fresh human tumors and human tumor cell lines. For fresh tumors, ID_{50} ratios for adriamycin, actinomycin D, and bisantrene ranged between 2 and 60 (median 14), whereas the ID_{50} ratios for bleomycin, vinblastine, and etoposide ranged between 100 and 3,000 (median 600). The fact that actinomycin D, adriamycin, and bisantrene (a new anthracene-type drug) had similarly shaped dose-response curves and very low ID_{50} ratios suggests that the cytotoxicity of these compounds may not be schedule-dependent. On the other hand, the steep dose-survival curves we observed after continuous drug exposure and the high ID_{50} ratios of bleomycin, vinblastine, and etoposide suggest that these drugs may possess schedule-dependent cytotoxicity characteristics. Before final conclusions are drawn concerning a drug's schedule dependency it is essential to evaluate its in vitro stability and protein-binding characteristics. Finally, it must be emphasized that unlike the results obtained with 1-h exposure studies, the in vitro continuous exposure schedules have yet to be shown to be predictive of clinical response for any agent or tumor type.*

Introduction

The efficacy of anticancer drugs may depend on the schedule of administration as well as the dose used. On the basis of studies in animal tumor models certain drugs, such as the bifunctional alkylating agents, are dose-dependent for their cytotoxicity [6, 22] and have been termed 'cell cycle-nonspecific' [23]. The cytotoxicity of agents, such as the antimetabolite cytosine arabinoside, appears to be schedule-dependent [10, 23], and these have been termed 'cell cycle-specific' [23]. There are limited data concerning the evaluation of drug schedule dependency in human tumor models. The in vitro human tumor clonogenic assay (HTCA) [11, 19] lends itself to the study of the schedule dependency of anticancer drugs [1, 3]. This assay has proven useful in accurately predicting clinical response to anticancer drugs [2, 18–20, 24]. The purpose of the present study was to evaluate the schedule dependency in vitro of various anticancer drugs using both fresh human cancers and human tumor cell lines in the HTCA.

Materials and methods

Fresh human tumor samples. Tumors from 174 patients were analyzed in 410 in vitro assays. The tumor types were: 47 ovarian, 33 melanoma, 15 breast, 14 lung, 13 bladder, 10 of unknown primary (adenocarcinoma), nine sarcoma, nine endometrial, seven kidney, three colon, two each of brain, stomach, pancreas, mesothelioma, and single cases of myeloma, testicular, pharyngeal and liver cancers. Cell suspensions were prepared according to techniques previously reported [11, 19].

Drugs. Stock solutions of IV formula adriamycin (Adria Laboratories, Columbus, OH), actinomycin D (Merck, Sharp & Dohme, West Point, PA), *cis*-diamminedichloro-platinum II (*cis*-platinum) (Bristol Laboratories, Syracuse, NY), 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 sensitivity studies. For the 1-h exposure studies, the single cell suspensions from fresh human tumors and from the human tumor cell lines were incubated with the anticancer drugs at concentrations ranging from 0.001 μg/ml to 1.0 μg/ml for 1 h at 37° C, as shown in Table 2 [1, 3]. Thereafter, the cells were washed twice and prepared for culture. For the continuous exposure studies the drugs were added at twice the final concentration to the tumor cells and media in the upper agar layer (see below for culture method). For the human tumor cell lines and fresh human tumor samples the in vitro concentra-

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tions used for each study drug are shown in Table 1. In general, the continuous exposure concentrations were about 1/200 those of the 1-h concentrations, since continuous exposure studies lasted about 200 h. All samples of fresh tumors and cell lines were simultaneously studied with both 1-h and continuous drug exposure.

Human tumor cell lines. A series of four human tumor cell lines was studied. Table 2 gives the name, source, tissue type, medium of culture and method of harvesting used for each of the cell lines. Cell lines were cultured at 37° C in 95% humidity, 5% CO_2, and 20% O_2. Cells were harvested during exponential growth. Final cell numbers per agar plate were the following: 50,000 cells (8226), 40,000 cells (WiDR), 200,000 cells (U266), and 40,000 cells (HEC-1A).

Human tumor clonogenic assay. The clonogenic assay has been detailed previously [11, 18]. Briefly, the upper layer consists of CRML 1066 and 15% horse serum, the underlayer of enriched McCoy's 5A with 10% FCS without conditioned medium. All samples plated were examined by inverted microscopy on day 1 to assure that a good single cell suspension had been obtained. As a control for proliferation a series of control plates were fixed in 3% glutaraldehyde and stored in the refrigerator until the time of analysis and examined simultaneously with the growth specimens to assure the absence of tumor cell aggregates. Incubated plates with at least 30 colonies of $> 60\,\mu m$ in diameter were analyzed with an automated image analysis system (Bausch and Lomb, Omicon FAS II). Reduction in the number of colony-forming cells to 30% of control or less after drug exposure was applied as the

Table 1. In vitro drug concentration tested against human fresh tumors and human tumor cell lines

Drug	Experimental concentration (ng/ml)			
	Human fresh tumors		Human tumor cell lines	
	1 h	Continuous	1 h	Continuous
Vinblastine	100, 10*	5, 0.5, 0.05*	100, 10, 1	5, 0.5, 0.05
Bleomycin	1,000, 100*	50, 5, 0.5*	1,000, 100, 10	50, 5, 0.5, 0.005
Etoposide	1,000*	5*, 0.5	10,000, 1,000, 100	500, 50, 5, 0.5
Adriamycin	100*, 10	5, 0.5*	100, 10, 1, 0.1	5, 0.5, 0.05
Actinomycin D	10*, 1	0.1, 0.05*	1,000, 100, 10	5, 0.5, 0.05
cis-Platinum	100*, 10	5, 0.5*	100, 10, 1	5, 0.5, 0.05
Bisantrene	1,000*, 100	5*	1,000, 100, 10	500, 50, 5

* Concentrations used for determination of drug sensitivity (i.e., ≤ 30% survival of TCFUs)

Table 2. Human cell lines and culture conditions

Cell line	Source	Tissue	Medium[c] (+ 10% FCS, + 1% PS)	Harvesting
RPMI 8226	ATCC[a]	Myeloma	RPMI 1640	Manual
WiDR	ATCC	Colon	RPMI 1640	Trypsing-EDTA 0.25% (Gibco)
HEC-1A	Dr Jorgen Fogh MSKCC[b]	Endometrial	McCoy's 5A	Tyrode's (Gibco)
U266	K. Nillson Sweden	Myeloma	RPM1 1640	Manual

Cells are harvested after 3–6 days of growth in Falcon 75 tissue culture flasks
[a] ATCC, American Type Culture Collection, Rockville, MD
[b] MSKCC, Memorial Sloan-Kettering Cancer Center, New York, NY
[c] fetal calf serum; PS, penicillin (10,000 u/ml)-streptomycin (10 mg/ml) (Gibco)

Table 3. Activity of anticancer drugs against TCFUs from fresh human samples following 1-h and continuous exposures

Drug	Total no. of tumors tested	Sensitive[a] to both 1-h and continous No. (%)	Sensitive to 1-h/resistant to continuous No. (%)	Resistant to 1-h/sensitive to continuous No. (%)	
Vinblastine	77	6 (7)	2 (2)	6 (8)	
Bleomycin	25	0 (0)	2 (8)	4 (16)	
Etoposide	40	0 (0)	2 (5)	6 (15)	
Adriamycin	67	1 (1)	5 (7)	0 (0)	P 0.004[b]
Actinomycin D	35	2 (6)	1 (2)	1 (2)	
Cisplatin	83	1 (1)	1 (1)	2 (2)	
Bisantrene	83	3 (4)	20 (24)	0 (0)	

[a] Sensitive = ≤ 30% survival of TCFUs (see *Methods* for cut-off concentrations used)
[b] Students *t*-test

criterion of in vitro sensitivity at the specific dose limits studied.

Data analysis

The following criteria for in vitro sensitivity of cells to drugs were applied to the survival-drug concentration curves. In vitro tumor sensitivity was defined as a reduction in tumor colony-forming units (TCFUs) to 30% or less of the control value at clinically achievable drug concentrations [1, 3, 15, 18, 24]. Previous clinical trials in which the results of the HTCA were used to select single-agent chemotherapy showed that objective response rates were in the range of 70% when survival of TCFUs was 30% or less at low in vitro drug concentrations [15, 18, 24]. The specific concentrations for each study drug which were used to determine drug sensitivity are marked with an asterisk in Table 1.

For comparison of the relative efficacy of the two dosing schedules, a ratio was calculated of the drug concentrations which reduced growth of TCFU to 50% of the controls (ID_{50}) for each tumor and cell line tested as shown below.

$$ID_{50}\ \text{ratio} = \frac{ID_{50}\ (\text{1-h exposure})}{ID_{50}\ (\text{continuous exposure})}.$$

Statistical analysis of the ID_{50} ratios was performed using a Wilcoxon test [15].

Results

One-hour and continuous drug exposure data

Table 3 shows in vitro numbers of fresh tumors sensitive to 1-h and/or continuous exposure. Overall a high percentage (72%–95%) of tumors were resistant to both 1-h and continuous exposures. Sensitivity to both schedules was only achieved in a small percentage of tumors (0–7%); however, six tumors (8%) tested with vinblastine, four (16%) tested with bleomycin, and six (15%) tested with etoposide (VP-16-213)

Table 4. Activity of schedule-dependent anticancer drugs against TCFUs from fresh human tumor samples which showed sensitivity following 1-h and/or continuous exposure

Cell cycle-specific drugs	Tumors sensitive[a] to 1-h and/or continuous exposure	Tumors resistant to 1-h and sensitive to continuous exposure	Percent of sensitive tumors
Vinblastine	14	6	42
Bleomycin	6	4	66
Etoposide (VP-16)	8	6	75

[a] Sensitive = ≤ 30% survival of TCFUs (see *Methods* for drug doses)

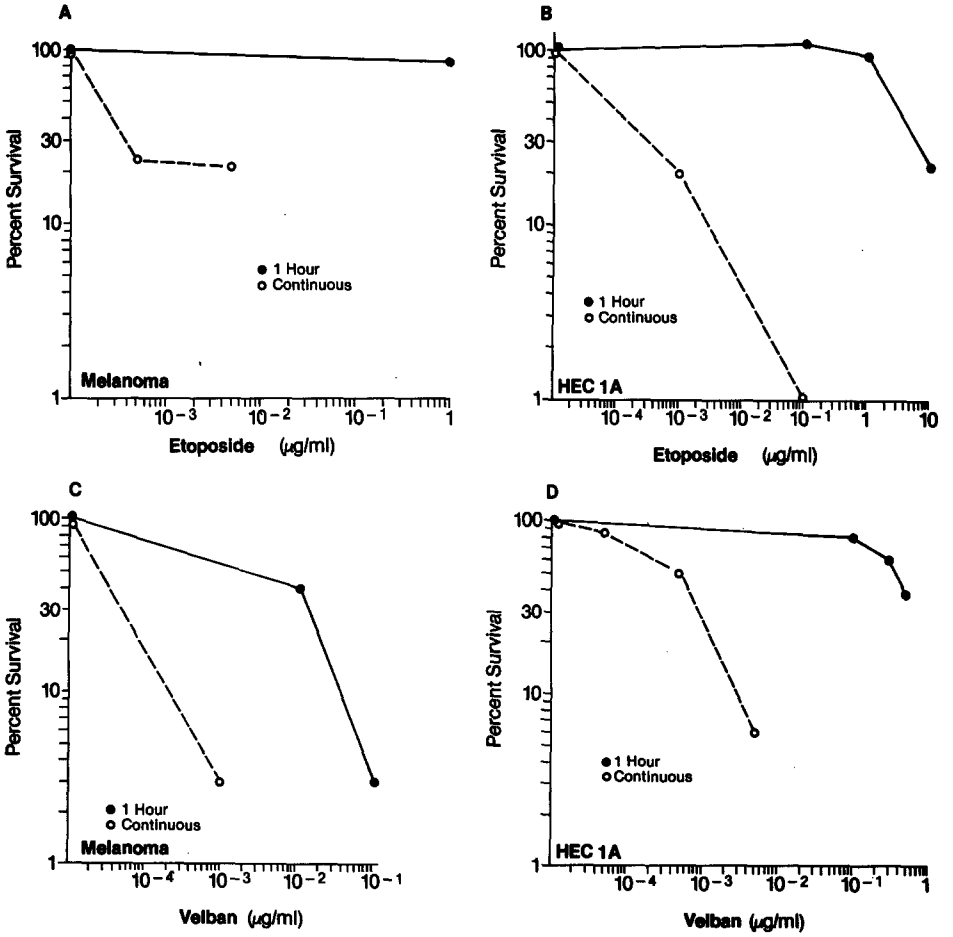

Fig. 1 A–D. Dose-survival curves for human TCFUs following 1-h and continous exposure to etoposide and vinblastine. **A** Fresh human melanoma TCFUs exposed to etoposide; **B** HEC-1A endometrial TCFUs exposed to etoposide; **C** Fresh human melanoma exposed to vinblastine; **D** HEC-1A endometrial TCFUs exposed to vinblastine

were resistant to 1-h but sensitive to continuous exposure at about 1/200 of the 1-h exposure concentration. In contrast, none, one (2%), two (2%), and none of the tumors were resistant to 1-h and sensitive to continuous exposure to adriamycin, actinomycin D, *cis*-platinum, and bisantrene, respectively. Five (7%) tumors tested with adriamycin and 20 (24%) tested with bisantrene were sensitive to 1-h and resistant to continuous exposure.

Table 4 shows data for tumors which were sensitive to either the 1-h or the continuous drug exposure schedules. For vinblastine, bleomycin, and etoposide, six of 14 (42%), four of six (66%), and six of eight (75%) tumors, respectively, were sensitive to continuous and resistant to 1-h exposures. In contrast, only one tumor tested with adriamycin, two tested with *cis*-platinum, and none tested with actinomycin D or bisantrene were resistant to 1-h and sensitive to continuous exposure.

Survival relationships

Figures 1 and 2 show dose-survival curves for the inhibitory effects of etoposide, vinblastine, adriamycin, and bisantrene on the growth of fresh human tumors and human tumor cell lines. Note that both etoposide (Fig.1A and B) and vinblastine (Fig. 1C and D) caused dose-related reduction in TCFUs from fresh tumors and cell lines when used in continuous exposure. One-hour incubations with these drugs failed to cause exponential reduction in TCFUs at the doses tested. In contrast, both 1-h and continuous exposure of these tumors to adriamycin (Fig. 2A and B) and bisantrene (Fig. 2C and D) resulted in similarly shaped dose-survival curves for TCFUs from fresh human tumors and human tumor cell lines.

Inhibitory dose 50 (ID_{50}) ratios

Figure 3 shows the comparative ID_{50} ratios (1-h versus continuous drug exposure) for the fresh human tumors exposed to adriamycin, actinomycin D, bisantrene, bleomycin, vinblastine, and etoposide. Note that the ID_{50} ratios for adriamycin, actinomycin D, and bisantrene ranged between 2 and 60 (median 14), whereas the ID_{50} ratios for bleomycin, vinblastine, and etoposide ranged between 100 and 3,000 (median 600). Figure 4 shows the comparative ID_{50} ratios (1-h versus continuous drug exposure) for the human tumor cell lines exposed to the same six drugs. The ID_{50} ratios for adriamycin, actinomycin D, and bisantrene ranged between 1.2 and 166 (median 33), and those for bleomycin, vinblastine, and etoposide, between 350 and 10,000 (median 3,125). The statistical analysis of the two medians showed a significant difference ($P \leq 0.01$) [16].

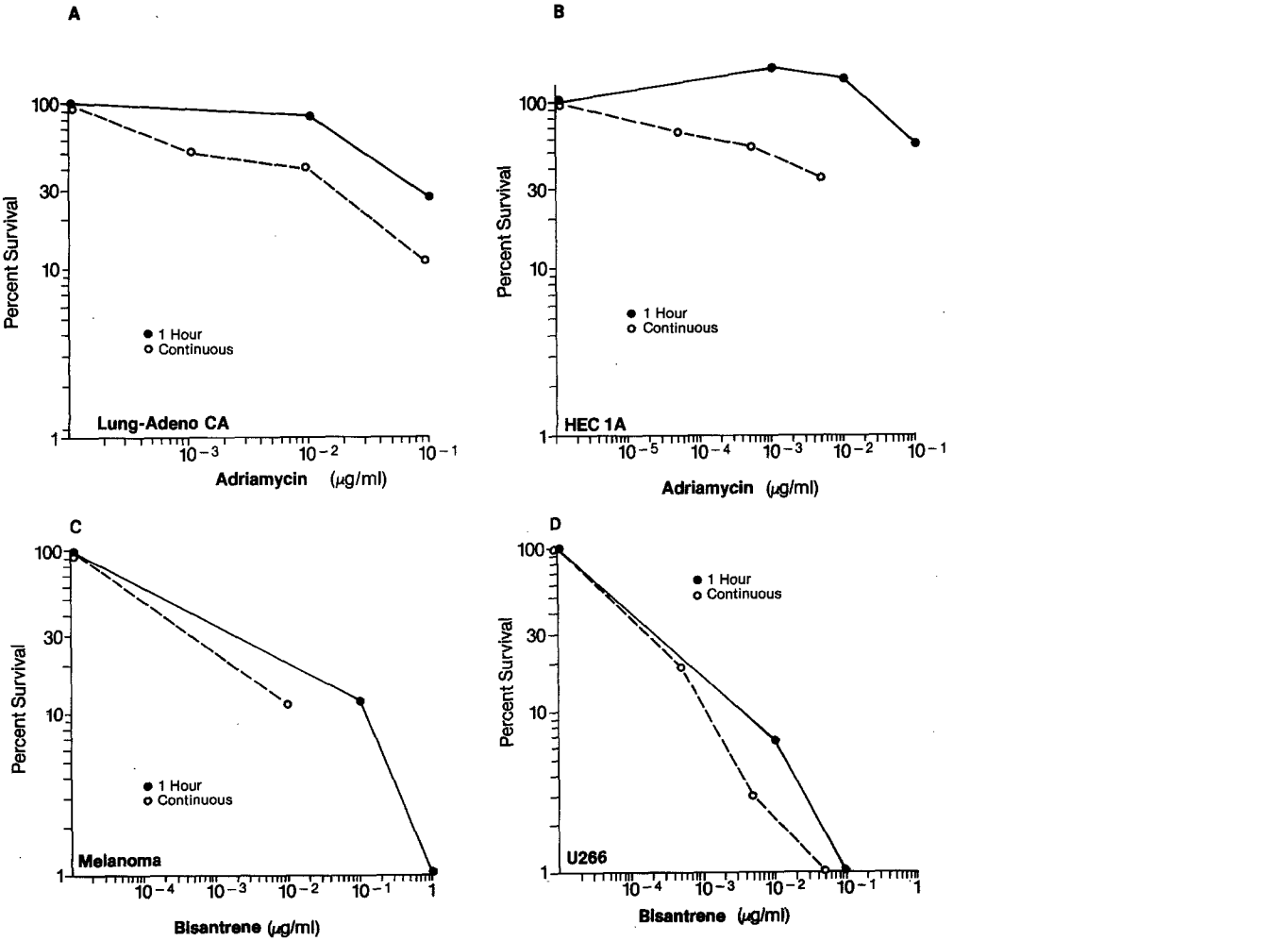

Fig. 2 A–D. Dose-survival curves for human TCFUs following 1-h and continous exposure to adriamycin and bisantrene. **A** Fresh human lung cancer TCFUs exposed to adriamycin; **B** HEC-1A endometrial TCFUs exposed to adriamycin; **C** Fresh human melanoma TCFUs exposed to bisantrene; **D** U266 myeloma TCFUs exposed to bisantrene

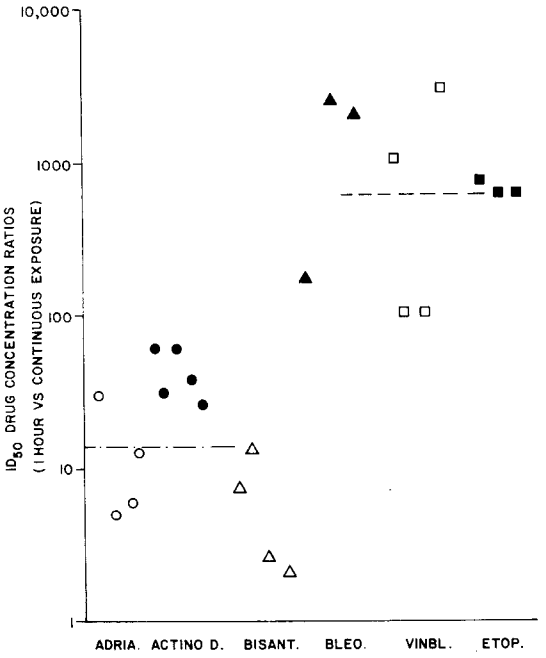

Fig. 3. Comparative ID_{50} ratios (1-h vs continuous for human fresh tumors exposed to various anticancer drugs. – · – · –, median ID_{50} ratio for adriamycin (Adria., ○), actinomycin D (Actino D., ●), and bisantrene (Bisant., △); ———, median ID_{50} ratio for bleomycin (Bleo., ▲), vinblastine (Vinbl., □), and etoposide (Etop., ■)

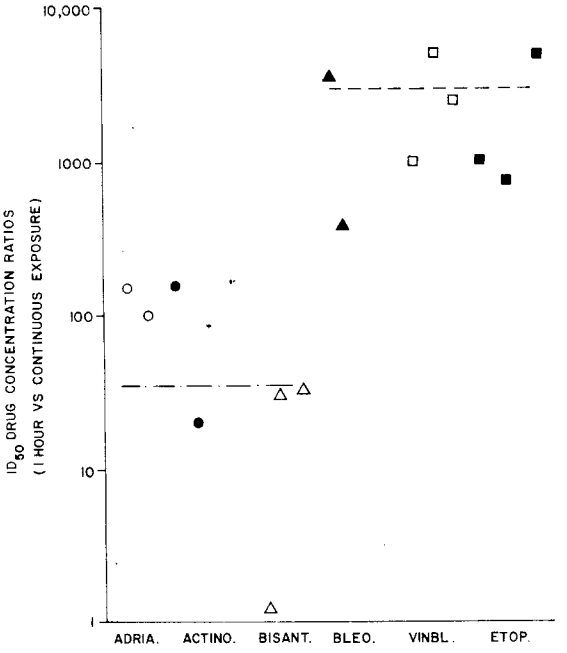

Fig. 4. Comparative ID_{50} ratios (1-h vs continuous) for human tumor cell lines exposed to various anticancer drugs. – · – ·–, median ID_{50} ratio for adriamycin (Adria., ○), actinomycin D (Actino., ●), and bisantrene (Bisant., △); ———, median ID_{50} ratio for bleomycin (Bleo., ▲), vinblastine (Vinbl., □), and etoposide (Etop., ■)

Discussion

Our studies suggest that the HTCA may be used to study the schedule dependency of standard and experimental anticancer drugs. By comparing the inhibitory effect on TCFU growth of 1-h and continuous drug exposures in the HTCA we were able to identify and separate schedule-dependent and schedule-independent drugs. These in vitro human tumor studies, using a clonogenic assay, serve to extend the studies of Bruce et al. [6], who evaluated the dose and schedule-dependent cytotoxicity of various anticancer drugs, using an in vivo murine lymphoma spleen colony assay. The drugs used in the present study were divided into two separate groups based on their schedule dependency. Three drugs (i.e., vinblastine, bleomycin, and etoposide), which are known to have cell cycle-specific characteristics [5, 8, 9], caused exponential reductions in tumor colony formation when given by continuous exposure, whereas when given with a short exposure each drug caused plateau-type curves. One possible explanation of these experimental observations is that prolonged drug incubation allows an increasing fraction of the clonogenic cells to enter the cell cycle and be exposed in their most sensitive phase to the cell cycle-specific agent, resulting in schedule-dependent cytotoxicity.

Our studies suggest that 1-h incubations of bleomycin, vinblastine, and etoposide may cause cytotoxicity to be underestimated in the clonogenic assay. Of our sensitive fresh human tumors, 42%–75% might have been incorrectly identified as 'resistant' to these drugs if the 1-h exposure had been the only schedule tested. Thus, it may be necessary to test these three schedule-dependent agents in the HTCA by continuous exposure for a more accurate assessment of their antitumor activity. However, caution must be used in interpreting the results of such studies. Whereas sensitivity of TCFUs to 1-h exposure reflects true drug-related lethality in that the observed growth inhibition persists in the absence of drug, sensitivity of TCFUs to continuous drug exposure does not allow one to distinguish between cytotoxic and cytostatic drug effects. Furthermore, there have been inadequate numbers of in vivo correlations with the in vitro continuous drug exposure experiments to document fully their clinical significance.

Comparison of dose-response data resulting from 1-h and continuous drug exposures in the HTCA may help identify whether a new anticancer drug is likely to possess schedule dependency for its cytotoxic effects [1, 3, 12]. For example, bisantrene, a new anthracene-type intercalating agent, has recently undergone phase-I trials on daily [21], weekly [4], and monthly [25] dosing schedules. Prior to these trials there were not adequate in vitro or in vivo data to suggest the most efficacious schedule for human testing. The fact that bisantrene had similarly shaped dose-response curves and a very low ID_{50} ratio after 1-h and continuous drug exposures suggests that its cytotoxicity is not schedule-dependent and should be most active clinically when used in a high dose with an intermittent schedule. On the other hand, the steep dose-survival curves which we observed after continuous drug exposure and the high ID_{50} ratios for 1-h versus continuous exposure testing of bleomycin, vinblastine, and etoposide against TCFUs from fresh solid cancers suggest that these drugs would ideally be used clinically by either continuous infusion (i.e., bleomycin and vinblastine) or daily dosing (i.e., etoposide). Indeed, the results of recent clinical trials in breast cancer with vinblastine [26], in cervix cancer with bleomycin [13], and in small cell cancers of the lung with etoposide [7] suggest enhanced activity for these drugs when they are given by continuous infusion (i.e., vinblastine and bleomycin) or daily (i.e., etoposide) dosing.

It is of interest that the data on schedule dependency for the drugs tested were similar in all cases in studies carried out

with both cell lines and fresh human tumors. This suggests that human tumor cell lines can be used to evaluate schedule dependency of new anticancer drugs and to gain some preliminary idea of their possible mode of action (i.e., cell cycle-specific versus cell cycle-nonspecific). Of course, more detailed studies using other methodologies are needed to define their precise mode of action and cell cycle phase specificity. If cell lines are selected which are relatively sensitive to an agent, then data concerning schedule dependency can be obtained rapidly. However, the fresh human tumor studies are important to not only confirm drug schedule dependency, but also to identify which tumor types are most likely to prove sensitive in phase-II trials.

We recognize that other factors may influence results in these in vitro studies of drug schedule dependency. If an anticancer drug is unstable in the in vitro cell culture system then possible schedule dependency characteristics may be blurred because of a rapid disappearance from the incubation medium. Similarly, unstable or highly protein-bound drugs may not be evaluated accurately in vitro for schedule dependency. For example, bisantrene has a high degree of protein binding in vivo and in vitro [16] and its low ID_{50} ratios for the 1-h and continuous exposure experiments could be related to this physiochemical phenomenon as well as its apparent lack of schedule dependency. Thus, before final conclusions are drawn concerning a drug's in vitro schedule dependency it is essential to evaluate its in vitro stability and protein-binding characteristics. The results of physiochemical studies of six of the seven agents evaluated in the present report are described in the companion paper in this journal [14]. Of these six compounds, actinomycin D, adriamycin, bisantrene, bleomycin, and vinblastine retained their biological (i.e., inhibition of TCFUs from human tumor cell lines) and chemical stability for up to 10 days in the standard HTCA culture media. Only etoposide showed evidence of degradation both biologically and chemically (HPLC assay) with about 40% of its initial concentration remaining after 72 h of incubation. In light of this instability etoposide's apparent schedule-dependent characteristics in the HTCA appear even more impressive than are suggested by the biological data included in the present report.

Finally, we must caution the reader that unlike the results obtained with 1-h exposure studies, the in vitro continuous exposure schedules have yet to be shown to be predictive of clinical response for any agent or tumor type. Definitive 'cut-off' concentrations for continuous exposure studies can only be firmly established when they are shown to predict response without an excessively high false-positive rate.

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