Eur J Cancer Clin Oncol. Vol. 19, No. 2, pp. 263–270, 1983.
Printed in Great Britain.

0277-5379/83/020263-08$03.00/0

Activity of Camptothecin, Harringtonin, Cantharidin and Curcumae in the Human Tumor Stem Cell Assay

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Abstract—*The antitumor activity of four investigational natural products (camptothecin, harringtonin, cantharidin and curcumae) obtained from China were tested on human tumor biopsies in an* in vitro *soft agar clonogenic assay system. Significant antitumor activity was seen with camptothecin against human ovarian cancer and some other adenocarcinomas. Antitumor activity was also observed for harringtonin against adenocarcinoma and sarcoma. Both drugs also appeared to show activity in melanoma and mesothelioma. However, cantharidin and curcumae were relatively ineffective on the human tumors tested. For purposes of comparing the intensity of antitumor effects with standard cytotoxic drugs to those of the four new agents, the* ID_{50} *values were calculated. The ratio of* ID_{50}*s of new drugs to the standard agents doxorubicin,* cis*-platinum and vinblastine (*ID_{50} *of the standard drug/*ID_{50} *of tested drug) were* 10.2, 64.1 *and* 1.9 *for camptothecin and* 1.5, 10.3 *and* 0.9 *for harrington respectively. A relationship was observed between the duration of drug exposure (*1 hr *prior to plating vs continuous contact in the agar) and inhibition of clonogenic tumor cells for camptothecin, harringtonin and doxorubicin.*

INTRODUCTION

AVAILABILITY of techniques to predict clinical chemosensitivity of anticancer drugs has particular importance for new agents. Recently, an *in vitro* soft agar clonogenic assay system (the human tumor stem cell assay) was developed [1–6]. It has proven useful for predicting the response of patients to chemotherapy. Clinical correlations thus far reported have shown a very high true-negative rate (failure of clinical response) of 95%, and a good true-positive rate (achievement of at least 50% tumor shrinkage) in 60–70% of patients with a wide variety of forms of cancer [1, 5, 6]. In view of its apparent clinical relevance and technical simplicity, the human tumor stem cell assay seems to have potential for application in discovering and developing new anticancer drugs. Steps at which the assay can potentially be useful include (a) preclinical drug screening for active compounds, (b) targeting tumor types for clinical trials of new agents and (c) the final selection of treatment for individual patients. With regard to drugs which have passed preclinical toxicology testing and are new in phase I–II clinical trials, the *in vitro* assay has several major values: it can potentially identify the antitumor spectrum of drugs and, in addition, by simultaneous comparison with standard drugs, provide some idea of relative activity in relation to known agents.

In recent years, research in China has placed a greater emphasis on natural products as sources for new antitumor agents [7, 8]. Several new agents from plants or animals are in clinical trial. In the present study we have used the human tumor stem cell assay to test four natural products which are currently in clinical trial in China. Several schedules of *in vitro* drug administration, 1-hr exposure and continuous contact of cells to drugs were used. Simultaneously, three standard drugs were used as 'positive controls' in order to contrast their lethality on clonogenic tumor cells to the new agents. The results indicate that camptothecin and harringtonin have significant

Accepted 23 August 1982.
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in vitro antitumor activity in the stem cell assay against human ovarian carcinoma and other adenocarcinomas as well as sarcomas.

MATERIALS AND METHODS

Four natural product antitumor drugs were obtained from the Institute of Chinese Meteria Medica in Beijing: camptothecin, an alkaloid isolated from *Camptotheca accuminata* Decne.; harringtonin, an ester-containing alkaloid isolated from *Cephalotaxus hainanesis* Li.; sodium cantharidin ('cantharidin'), a derivative of cantharidin which was isolated from the beetle *Mylabris phalerata* Pall.; and the oil of curcumae ('curcumae'), an essential oil from *Curcumae wenchowensis* Sp. Nor. All were water-soluble except for curcumae, which was prepared as an oil-based solution with a solubilizer added so that it could be dissolved in low concentrations in sterile distilled water. All drug dilutions were stored at -80°C in small aliquots until the time of *in vitro* testing. Standard anticancer drugs which were included for comparison included an alkylating agent (*cis*-platinum), an antibiotic (doxorubicin) and a plant alkaloid (vinblastine). These standard agents were tested simultaneously on the same tumor specimens as the new agents. Each drug was tested at three dose levels.

Patient specimens

Solid tumor biopsies and malignant effusions from a variety of human carcinomas or sarcomas were utilized in these studies. Tumor cells were studied from 12 patients with ovarian, four lung, four cervical or uterine carcinomas, four other adenocarcinomas, and two patients each with melanoma, mesothelioma and sarcoma. Eleven of the 30 patients had previously received chemotherapy (6 ovarian, 1 lung, 1 uterus, 1 pleural adenocarcinoma, 1 mesothelioma, 1 sarcoma).

In vitro *clonogenic assay*

Two schedules of drug exposure were tested: 1-hr exposure prior to plating, and continuous exposure in the agar. All drugs were tested at three concentrations with both testing schedules. For 1-hr exposure 1.5×10^6 tumor cells were incubated with and without the drug for 1 hr at 37°C in McCoy's 5A medium with 10% fetal calf serum (FCS). The cells were then washed twice with McCoy's 5A and prepared for culture. For continuous contact the drugs were incorporated into the upper agar layer for the duration of culture.

The clonogenic assay system utilized was a minor modification of the method of Hamburger and Salmon [2]. Conditioned medium was not required.

For each assay six control plates along with triplicate plates for each concentration of each drug exposure were employed for each tumor specimen tested. One milliliter containing 5×10^5 cells in 0.3% agar in enriched CMRL 1066 medium was plated over the 0.5% agar feeder layer in each 35-mm Petri dish. Plates were cultured in 5% CO_2 in air at 37°C in a humidified incubator. Colony growth was checked twice weekly. Colonies were usually present in sufficient numbers for counting 14–21 days (average 18 days) after plating. Only specimens with good *in vitro* growth were utilized. At least 30 tumor colonies per control plate (average: 72 with 1-hr exposure, 77 with continuous contact) were required for a drug experiment to be considered evaluable for measurement of drug effect. Thus the average relative cloning efficiency was in the range of 0.01%. Fresh tumor cell suspensions contained varying numbers of host and tumor cells, and absolute cloning efficiencies were not routinely determined. Colony counting was carried out with an automated image analysis system (Bausch and Lomb Omnicon FAS II). Representative plates were prepared for morphologic analysis using a dried-slide technique with Papanicolau staining [9]. The mean coefficient of variation in control plates was 22 ± 12%.

The following two criteria for *in vitro* sensitivity of cells to drugs were applied to the colony survival drug concentration curves: (1) a 70% reduction in the survival of tumor colony-forming units (TCFU) at a relatively low drug dose was operationally defined as sensitivity, as it relates to the cut-off used in major clinical trials that have related *in vitro* and *in vivo* sensitivity [10]; and (2) for comparison of the relative efficacy of two schedules or two drugs, the drug concentration which would inhibit growth of TCFU to 50% of the control (ID_{50}) was calculated from the survival curves. The ID_{50} value has commonly been used to report results of *in vitro* studies of cellular sensitivity to anticancer drugs and is therefore provided for comparative purposes. Because of the lack of accurate pharmacokinetic data for the new agents, fixed ratios to the clinical trial doses of these drugs were utilized and the low dose level for the agents was arbitrarily set at 0.1, 0.1, 0.01 and 0.3 μg/ml with camptothecin, harringtonin, cantharidin and curcumae respectively (1-hr exposure).

RESULTS

Drug assays

Camptothecin sensitivity. Survival plots of tumor colony-forming cells after continuous contact or 1-hr exposure to camptothecin are

shown in Fig. 1. Reduction in survival to 30% of control TCFU at the tested concentrations (0.0001–0.1 μg/ml) in the continuous contact experiments was observed on each tumor type except lung cancer and cervical carcinoma. There was definite lethality in cells from 11 of the 26 patients, which included 4 of the 10 patients with ovarian carcinoma (at the 0.01 μg/ml concentration level). With lower concentrations of camptothecin, sensitivity of TCFU was still observed in 5 of 26 and 2 of 12 patients at 0.001 and 0.0001 μg/ml respectively. In the 1-hr exposure experiments, survival of TCFU for 2 of 5 patients with ovarian cancer was also reduced to 30% of control at concentrations of 0.1 and 0.01 μg/ml.

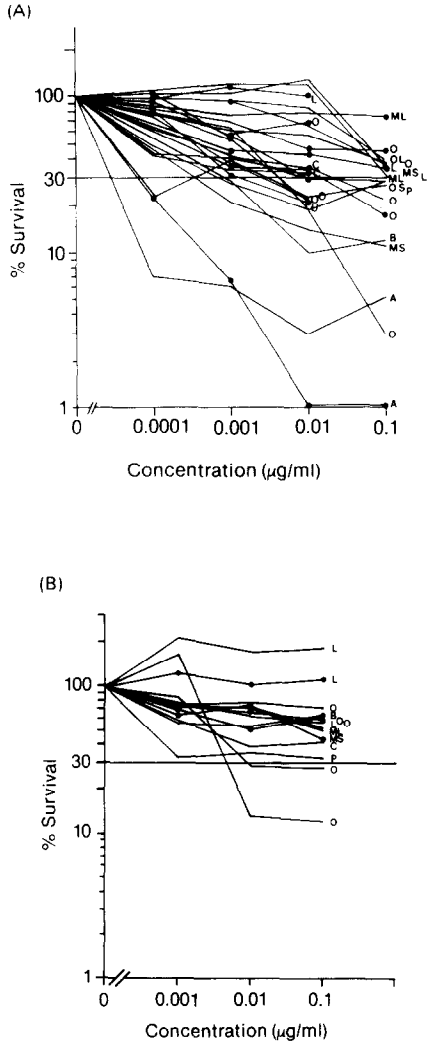

Fig. 1. Survival of TCFU after in vitro *exposure to camptothecin. (A) Continuous exposure of cells to the drug. (B) One-hour exposure of cells to the drug. Closed circles are data points for patients who were previously untreated, whereas unaccented curve inflexions and end points represent data points for patients who had received prior chemotherapy. O, Ovarian; L, lung; A, adenocarcinoma; P, pancreas; B, breast; U, uterus; C, cervical carcinoma; MS, mesothelioma; ML, melanoma; S, sarcoma.*

Harringtonin sensitivity. Survival curves with harringtonin on tumor biopsies from 30 patients are shown in Fig. 2. The steepening of curves and heterogeneity of tumor sensitivity to harringtonin are more obvious than that of camptothecin. With continuous exposure harringtonin caused a reduction in survival to 30% of control in cells from 18 of 28, 10 of 27 and 3 of 28 cases at the concentrations of 0.1, 0.01 and 0.001 μg/ml respectively. The spectrum of sensitive tumors included sarcomas and adenocarcinomas. With the 1-hr exposure marked inhibition of tumor colony formation was noted in one pancreatic adenocarcinoma at the 0.01 μg/ml concentration, and one case each of ovarian and cervical carcinoma at the 0.1 μg/ml dosage.

Cantharidin sensitivity. Tumor cells from 19 patients with nine types of cancer were tested with cantharidin over a wide concentration range (Fig. 3). In most instances only two tumors (ovarian

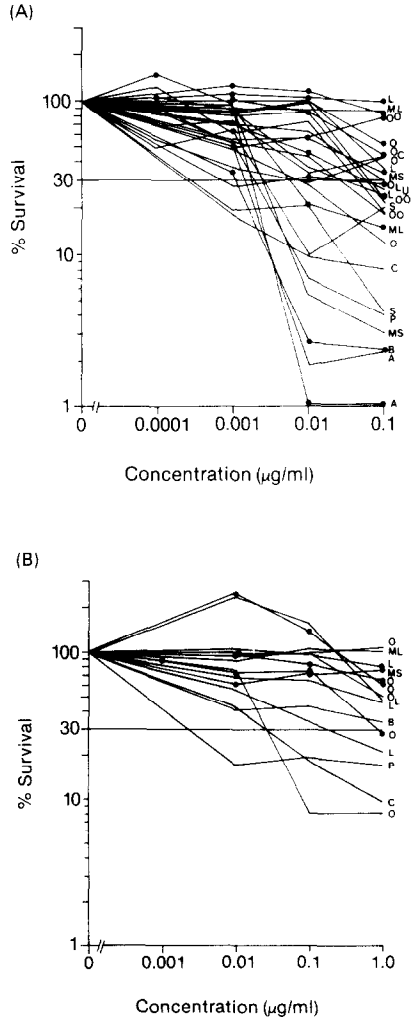

Fig. 2. Survival of TCFU after in vitro *exposure to harringtonin. (A) Continuous exposure of cells to the drug. (B) One hour exposure of cells to the drug. Definitions were as detailed in Fig. 1.*

and melanoma) manifested sensitivity to cantharidin at the 0.1 μg/ml concentration.

Curcumae sensitivity. A total of 19 specimens from patients with nine types of cancer were evaluated for sensitivity to curcumae in the 1-hr exposure and continuous-contact experiments (Fig. 4). None of 10 samples showed any sensitivity in continuous contact. In almost all instances survival of TCFU was more than 70% of the control. In one case there was a positive relation between concentration of the drug and survival of TCFU. On the other hand, one sample from a pancreatic carcinoma manifested definite sensitivity to the drug in 1 of 9 1-hr exposure experiments.

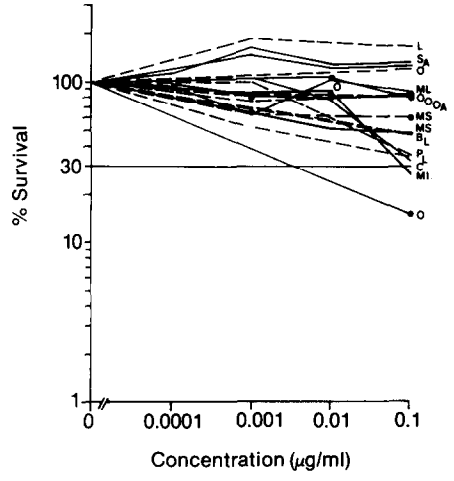

Fig. 3. Survival of TCFU after in vitro *exposure (—— continuous exposure, ----- 1-hr exposure) to cantharidin. Definitions were as detailed in Fig. 1.*

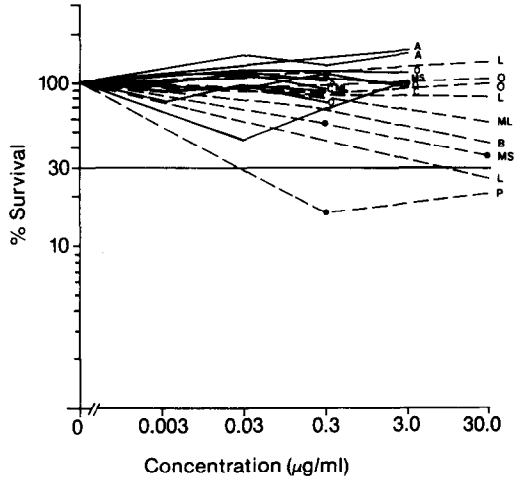

Fig. 4. Survival of TCFU after in vitro *exposure (—— continuous exposure, ----- 1-hr exposure) to curcumae. Definitions were as detailed in Fig. 1.*

Comparative activity of new agents and standard cytotoxic drugs

In order to better assess the activity of the new agents tested, three standard cytotoxic drugs, doxorubicin, *cis*-platinum and vinblastine, were also tested simultaneously at similar concentrations on aliquots from the same biopsy specimen. The survival plots for continuous contact experiments with these standard drugs are shown in Figs 5, 6 and 7. The drug concentration which caused 50% decrease in TCFU (ID_{50}) was calculated. Individual ID_{50} ratios were calculated for each tumor tested with the investigational and standard agents tested. Table 1 lists the median ID_{50} ratios of camptothecin or harringtonin and the three standard drugs. Based on this comparison, the lethality of 0.01 μg/ml of camptothecin appears similar to that attained with 0.10 μg/ml of doxorubicin. Based on the ID_{50} ratios, harringtonin had similar activity to vinblastine in the continuous contact experiments.

Relation of drug schedule to lethality on clonogenic tumor cells

Exposure of tumor cells to drugs for 1 hr before plating and continuous contact of the cells with the drug in agar were evaluated as two different drug schedules. Individual ID_{50} ratios were calculated for each tumor tested simultaneously with each drug by 1-hr and continuous exposure *in vitro*. By comparing the ID_{50} ratios of 1-hr exposure and continuous contact for each of the biopsy specimens tested (5 ovarian, 3 lung and 1 each of pancreatic, cervical carcinoma, melanoma and mesothelioma), we found that the median of ID_{50} ratios (1-hr/continuous) were quite similar for camptothecin, harringtonin and doxorubicin (Table 3), with values in the range of 7.5–10. When we compared continuous exposure dosages of camptothecin, harringtonin or doxorubicin that were 1/10 of the 1-hr concentrations, then identical average inhibition rates were obtained for each of these agents (Table 3).

Based on these data we chose to select concentrations of 0.01, 0.01, 0.001 and 0.03 μg/ml respectively for camptothecin, harringtonin, cantharidin and curcumae as the boundary dosage levels at which to determine sensitivity of TCFU to those drugs in the continuous contact experiments. The results of sensitivity of the new drugs and several standard agents tested with the two dosage schedules are summarized in Table 4. Computations of the frequency of sensitivity of the new agents must be considered as preliminary and would require extended testing on a larger number of tumor specimens as well as correlative clinical trials for validation. The sensitivity of

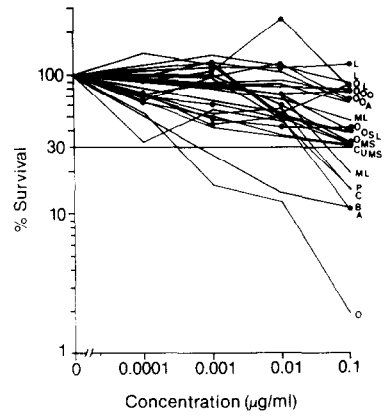

Fig. 5. Survival of TCFU after in vitro *continuous exposure to adriamycin. Definitions were as detailed in Fig. 1.*

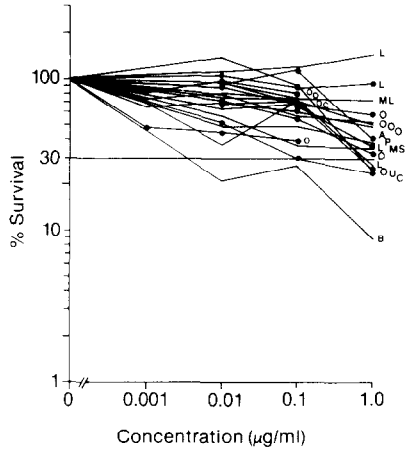

Fig. 6. Survival of TCFU after in vitro *continuous exposure to cis-platinum. Definitions were as detailed in Fig. 1.*

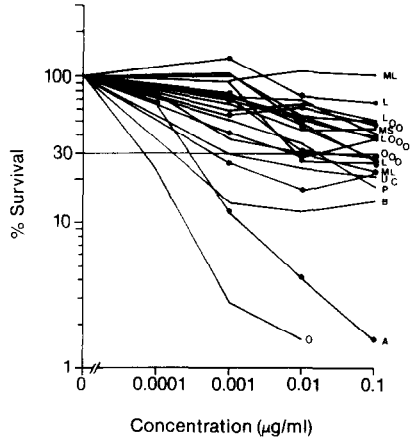

Fig. 7. Survival of TCFU after in vitro *continuous exposure to vinblastine. Definitions were as detailed in Fig. 1.*

ovarian TCFU to doxorubicin and *cis*-platinum was less than that observed in our laboratory in prior experiments and may relate to the limited sample size of ovarian tumor specimens from patients who had not received prior therapy with these agents.

DISCUSSION

Camptothecin, harringtonin, cantharidin and curcumae are currently under evaluation in clinical trials in China. Camptothecin exhibited definite antineoplastic activity against animal tumors and has been subjected to a number of *in vitro* studies of its cellular cytotoxicity [11–13]. Camptothecin was also tested for treatment of gastric carcinoma and other malignancies in the United States [14–16]. However, because of toxic side-effects (e.g. hemorrhagic cystitis), the United States camptothecin clinical trials were discontinued by the National Cancer Institute (NCI). Von Hoff has previously commented on the potentially premature discontinuation of camptothecin and other phase I–II agents (because of early difficulties with excessive toxicity, preliminary conclusions of lack of efficacy or simple neglect [17]). With regard to bladder toxicity, an analogous problem to that observed with camptothecin had been observed with the alkylating agent, isophosphamide [18]. Recent European trials of sodium-2-mercaptoethane sulfonate (MESNA) have apparently markedly reduced the frequency of hemorrhagic cystitis with isophosphamide [18], and antidotes for drugs such as camptothecin may be available. In China, camptothecin is currently in use with

Table 1. ID_{50} *ratios of camptothecin or harringtonin and three standard drugs*

	Doxorubicin	*cis*-Platinum	Vinblastine
Camptothecin	10.2 (22)	64.1 (19)	1.9 (17)
Harringtonin	1.5 (22)	10.3 (19)	0.9 (18)

The data listed are median of ID_{50} ratio between two drugs (ID_{50} of standard drugs/ID_{50} of camptothecin or harringtonin). The number of samples tested is shown in parentheses.

Table 2. Ratio of ID_{50} *(1-hr exposure/continuous contact) of camptothecin, harringtonin and doxorubicin between two schedules*

	Camptothecin	Harringtonin	Doxorubicin
Cases	9	11	8
Mean	17.9	18.9	18.6
Median	9.9	7.5	10.0
S.E.	9.9	8.8	9.8

Table 3. Comparative survival of 1-hr and continuous contact schedules of camptothecin, harringtonin and adriamycin

Drug	No. of cases tested	1-hr exposure Concentration (μg/ml)	1-hr exposure Survival (%)	Continuous contact Concentration (μg/ml)	Continuous contact Survival (%)
Camptothecin	13	0.1	62.8 ± 11.3	0.01	62.3 ± 10.2
Harringtonin	12	0.1	74.2 ± 11.2	0.01	67.8 ± 9.2
Adriamycin	13	0.1	85.4 ± 12.5	0.01	86.0 ± 16.2

Table 4. Frequency of in vitro *sensitivity* of human tumor stem cells to new agents and standard drugs tested*

Tumor type	Continuous contact							1-hr exposure				
	Camptothecin	Harringtonin	Cantharidin	Curcumae	Doxorubicin	*cis*-Platinum	Vinblastine	Camptothecin	Harringtonin	Cantharidin	Curcumae	Doxorubicin
Ovarian	4/10†	1/11	0/3	0/2	1/10	0/10	3/9	2/5	1/6	0/2	0/2	0/5
Lung	0/4	0/4			0/4	0/4	0/4	0/4	0/4	0/3	0/3	0/4
Adenocarcinoma	2/2	2/2	0/1	0/1	0/2	0/1	1/1					
Pancreas	0/1	1/1			0/1	0/1	0/1	0/1	1/1	0/1	1/1	1/1
Breast	1/1	1/1			1/1	1/1	1/1	0/1	0/1	0/1	0/1	0/1
Uterus	1/1	0/1			0/1	1/1	1/1					
Cervical	0/2	1/2	0/1	0/2	0/2	0/2	1/1	0/1	1/1			0/1
Mesothelioma	1/2	1/2	0/1	0/1	0/2	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Melanoma	1/2	1/1	0/1	0/1	0/2	0/1	1/2	0/1	0/1	0/1	0/1	0/1
Sarcoma	1/1	2/2	0/1	0/1	0/1							
Total	11/26	10/27	0/8	0/8	2/26	2/22	8/21	2/14	3/15	0/9	1/9	1/14

*Sensitivity criteria are tentative for the new agents (see text).
†Number of sensitive cultures/No. tested.

efforts to find the appropriate dose or schedule of administration and thereby improve its therapeutic index. Additionally, the study of derivatives (e.g. monohydroxycamptothecin) and a search for antidotes to camptothecin toxicity (e.g. monoammonium glycyrrhizinate) have been pursued [15, 19, 20]. These studies have reported that camptothecin may have therapeutic value in gastric, liver and other cancers [7, 8]. A dosage of 0.2 mg/kg by continuous infusion for 10–15 days was considered clinically tolerable and was utilized in the Chinese trials. It is important to point out that our *in vitro* studies utilized the same formulation of the free drug camptothecin as is in use in China. In contrast, the phase I–II studies performed in the United States [14–16] used the sodium salt of camptothecin. When camptothecin is neutralized to form the sodium salt, the lactone ring is opened. As a result, its antitumor activity might be decreased and/or its toxicity to normal tissue (e.g. urinary bladder) might be increased. To address this question, direct comparison of these two formulations to assess their relative lethality against TCFU *in vitro* would be of value. In our *in vitro* studies reported in this paper it appeared that camptothecin had activity in 4/10 ovarian cancers at the continuous-contact dose of 0.01 μg/ml concentration. Our use of the plant alkaloid, vinblastine, as one of the standard drug controls for the camptothecin experiments provided a basis for some interesting comparisons of relative activity in ovarian cancer wherein both drugs were active *in vitro*. With camptothecin, only one-half the dose was required to achieve an ID_{50} as was needed with vinblastine. We speculate that a clinical trial of camptothecin in ovarian cancer using a low-dose continuous infusion (e.g. 8–10 mg/m^2 over one week rather than the usual higher dose of 44 mg/m^2) might prove efficacious.

Pharmacological experiments have shown that harringtonin can inhibit protein and nucleic acid synthesis in tumor cells and it also had antineoplastic activity against certain animal tumors [21, 22]. Clinically, this drug has been tested in China only against acute myeloblastic

leukemia, acute monoblastic leukemia and erythroleukemia, and it appeared to have a substantial therapeutic activity at the clinical dose of 0.2 mg/kg/day for 5–10 days [23]. *In vitro* data from the current study provided further indication that harringtonin might have activity in adenocarcinoma and sarcomas. This information may be of value for enlarging the antitumor spectrum of the drug in clinical investigation. An analog (homoharringtonin) has recently become available through the NCI in the United States and will be of interest to compare with the parent compound. Homoharringtonin will be entering clinical trial in the near future, and such *in vitro* comparisons may be of value.

Cantharidin and curcumae have been used in China mainly on hepatocarcinoma and cervical carcinoma. Their tumor-inhibiting effects were reported for animal tumors and in the clinical studies. Cantharidin interfered with the metabolism of nucleic acid and protein in cancer cells and curcumae enhanced host immunity to tumor tissue [8, 24, 25]. However, in our *in vitro* experiments these agents were ineffective except in one tumor tested for 1 hr with curcumae. There are several possibilities for this discrepancy. Firstly, the number and different types of specimens may have been inadequate to indicate whether certain tumor types would be sensitive to these drugs. Secondly, the drugs may require bioactivation *in vivo* to produce their antineoplastic effect. Thirdly, some drugs may act as immunostimulants and not be active in this assay system. Finally, it is quite possible that neither cantharidin nor curcumae has significant antitumor activity.

The procedure of continuous contact provides a broader test for new agents than 1-hr exposure as activity of cell cycle-specific agents is unlikely to go undetected. The NCI introduced the human tumor stem cell assay into their new drug screening program in 1980 and has used the continuous contact exposure as a first step for evaluating antitumor activity [26]. However, using the continuous exposure technique, it is not possible to distinguish between cytotoxic and cytostatic drugs. Sensitivity of TCFU to a 1-hr exposure does reflect true lethality as any growth inhibition observed persists in the absence of the drug. It is important to define the relationship of drug concentrations in 1-hr exposure and continuous-contact studies and determine how best to assess the appropriate concentrations to predict the *in vivo* sensitivity. Alberts *et al.* [1] found that the bleomycin caused markedly greater cell kill when given by continuous *in vitro* contact and suggested the concentration of a stable cycle-active drug would be less than 1/200 to 1/300 of the 1-hr exposure concentration for an accurate prediction of TCFU sensitivity to continuous drug contact. This formula is based on the total number of hours of exposure over the usual 8–13 days of culture required for ovarian cancer cell growth in agar. In contrast, 'cell cycle non-specific' agents did not show any distinct schedule dependency for their *in vitro* cytotoxic effects. Of the drugs we studied, camptothecin, harringtonin and doxorubicin can be considered to be cell cycle non-specific agents. The median of ID_{50} ratio of each drug between 1 hr and continuous was about 10, but the mean was about 18. The median is a preferable basis for comparison as it is insensitive to extreme values. The values of medians were similar to those found in comparison of average survival rate with two schedules. When considering the standard errors observed, we would suggest that drug concentrations in continuous exposure initially be in the range of 1/10–1/40 of the 1-hr exposure concentration. From the above, it is possible to recognize that the 'cell cycle-specific' and cell cycle 'non-specific' agents may have different values for their concentration ratios in 1-hr and continuous exposure studies. However, it is important to recognize that exposure time studies alone represent only preliminary assessments of cycle relatedness or schedule dependency of any given drug. Such leads must be followed up with more detailed studies of drug stability and cycle specificity using more precise analytic techniques.

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