PHIGENIX
Exhibit 1031

DOCKET ALARM
Find authenticated court documents without watermarks at docketalarm.com.

Cancer Research

Differential Killing Efficacy of Twenty Antitumor Drugs on Proliferating and Nonproliferating Human Tumor Cells

Benjamin Drewinko, Myra Patchen, Li-Ying Yang, et al.

Cancer Res 1981;41:2328-2333.

Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/41/6/2328

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

PHIGENIX
1031-01

[CANCER RESEARCH 41, 2328–2333, June 1981]
0008-5472/81/0041-0000$02.00

Differential Killing Efficacy of Twenty Antitumor Drugs on Proliferating and Nonproliferating Human Tumor Cells[1]

Benjamin Drewinko,[2] Myra Patchen, Li-Ying Yang, and Barthel Barlogie

Departments of Laboratory Medicine [B. D., L. Y. Y.] and Developmental Therapeutics [M. P., B. B.], The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77030

ABSTRACT

The lethal effects of a 1-hr treatment with 20 antitumor drugs on proliferating and nonproliferating cultured human colon carcinoma cells (line LoVo) were analyzed quantitatively by the colony-forming technique. Proliferating cells were obtained from exponentially growing cultures, while nonproliferating cells were from cultures in a stationary phase of growth. The 1-hr treatment was intended to approximate serum peak levels after bolus administration. Two agents, *cis*-platinum and vindesine, were more effective on nonproliferating than on proliferating cells. Mitomycin C, nitrosoureas, and dihydroxybisalkylanthracenedione were equally effective on proliferating and nonproliferating cells. The low lethal activity (<1 log) of methylglyoxal bis(guanylhydrazone), hycanthone, and vinblastine was similar in proliferating and nonproliferating cells. For most drugs (Adriamycin, rubidazone, bleomycin, maytansine, vincristine, epipodophyllotoxin, fluorouracil, hydroxyurea, methotrexate, and transplantinum) cytotoxicity was significantly less pronounced (or even totally absent) in nonproliferating than in proliferating cells. These results demonstrate the significance of cellular proliferation kinetics in determining sensitivity to antitumor therapy. Nonproliferating human cells have decreased sensitivity to most antitumor agents. An occasional agent may present increased activity to nonproliferating cells; but at best, few agents can be expected to be as effective on nonproliferating as on proliferating cells.

INTRODUCTION

Cell cultures provide a rapid, efficient, and economical assay system for cytotoxicity screenings of antitumor agents, allowing elucidation of the mode of action of a drug in a controlled systematic fashion with a high degree of resolution. However, a major drawback of tissue culture experiments resides in the difficulty of extrapolating information obtained on exponentially growing cells to the expected responses of tumor cells *in vivo* where large fractions of the population may be in the quiescent state (G_0 cells) (43). Quiescent cells are usually considered less sensitive than proliferating cells to the lethal activity of most antitumor drugs (3, 6, 9, 17, 37, 52, 54–56).

Recent investigations suggest the usefulness of utilizing stationary-phase cultures as an adequate *in vitro* model of *in vivo* neoplasias with low fractions of proliferating cells (low-growth fraction) (2, 3, 24, 32). Stationary-phase cultures are obtained by allowing cells to replicate without refeeding until such time when no net increment in cell numbers is demonstrated. This can result either from cessation of multiplication (with elongation of cell cycle transit times) or from a balance between cell death and cell multiplication (*i.e.*, the number of cells born per unit time equals the number of cells disintegrating per unit time). Some investigators have studied the lethal activity of antitumor agents on this type of culture system using cells of rodent origin. They reported considerable differences with respect to the response obtained for exponentially growing cultures (4, 5, 44, 46) and suggested that these differences might be exploited at the clinical level (49).

We evaluated the lethal efficacy of a variety of antitumor agents on an established colon carcinoma cell line of human origin while in the exponential and in the stationary phases of growth. Our results demonstrated that, in contrast to studies effected on rodent cells, human cells in the stationary phase of growth have decreased sensitivity to most antitumor agents.

MATERIALS AND METHODS

Cell Line. The cells used in this investigation were from a carcinoembryonic antigen-producing colon carcinoma line (LoVo cells) established in 1972 (12). Cells are maintained as monolayer cultures in Ham's F-10 medium supplemented with 20% fetal calf serum, vitamins, glutamine, and antibiotics. LoVo cells generate glandular structures *in vitro* when grown as monolayers and *in vivo* when propagated as xenografts in nude mice (14). The generation time of exponentially growing cells is 29.3 hr, and the transit times through each stage of the cell cycle are: G_1, 14.7 hr; S, 10.7 hr; and T_{G_2} + M, 4.8 hr. Single cells plated in fresh medium give rise to large colonies with a PE[3] ranging from 35 to 70% (14).

Growth Kinetics Characteristics. To establish the time sequence of the *in vitro* growth of LoVo cells (*i.e.*, exponential and stationary phase), aliquots of 10^5, 5×10^5, and 7×10^5 cells were seeded into sets of 60-mm Petri dishes containing 5 ml of growth medium and maintained without refeeding throughout the span of the experimental interval. Every 24 hr for 21 days, 2 replicate cultures from each set were harvested using techniques described previously (12) and were processed for cell counting, LI determinations, FCM studies, and colony formation. Cell counts were performed with the aid of an electronic particle counter (Model ZBI Coulter Counter; Coulter Electronics, Inc., Hialeah, Fla.). For LI determinations, the cells were pulse-labeled for 30 min with [^{3}H]dThd (1 µCi/ml; specific activity, 3.0 Ci/mmol) before harvesting. Cytocentrifuge preparations were processed for radioautography using a 50% solution of Ilford emulsion (Polysciences, Inc., Warrington, Pa.) in distilled water, exposed for 2 weeks, and developed

[1] Supported by Grants CA 23272, CA 14528, and CA 16763 from National Cancer Institute, Department of Health, Education, and Welfare.

[2] To whom requests for reprints should be addressed.

Received July 21, 1980; accepted March 5, 1981.

[3] The abbreviations used are: PE, plating efficiency; LI, labeling index; FCM, flow cytometry; dThd, thymidine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; BLEO, bleomycin; *cis*-DDP, *cis*-diamminedichloroplatinum; VDS, deacetyl vinblastine amide sulfate.

DOCKET ALARM
Find authenticated court documents without watermarks at docketalarm.com.
PHIGENIX
1031-02

in Kodak D19 (Eastman Kodak, Rochester, N. Y.). FCM studies were conducted on mithramycin-ethidium bromide-stained cells using a Phywe ICP II pulse cytophotometer (Phywe AG, Gottingen, Germany) (14). For PE determinations, aliquots of 200 cells/dish were dispensed into 60-mm Petri dishes, incubated for 21 days, stained, and fixed with 2% crystal violet in 95% ethanol, and the colonies were counted with a stereomicroscope. PE was defined as the ratio of colonies to the number of plated cells.

Survival Assay. Stock cultures were harvested and counted with the aid of a Model ZBI Coulter Counter electronic particle counter. Cell suspension aliquots were seeded into 60-mm Petri dishes (5×10^5 cells/dish). The cells were incubated at 37° in a 5% CO_2 atmosphere in air for 48 to 72 hr to achieve exponential growth and for 8 days without medium replenishment to achieve a stationary growth phase (see "Results"). The medium was discarded, and the cells were exposed to increasing drug concentrations for 1 hr at 37°. The drug was decanted, and the cells were washed twice in Hanks' balanced salt solution, harvested as a monodispersed suspension immediately after the treatment using hyaluronidase (10^2 IU/ml) for 5 min followed by trypsin (2.5%) for 5 min, and then counted. Known aliquots of the cell suspension were dispensed into 60-mm Petri dishes so that 50 to 100 colonies would appear after 21 days of incubation in a 5% CO_2 humidified atmosphere at 37°. The colonies were stained with 2% crystal violet in 95% ethanol. Viability was defined as the ability of single cells to give rise to a colony of ≥50 cells. In each experiment, the PE of at least 6 control cultures was assessed simultaneously. Control cultures, both in exponential and in stationary growth phases, consisted of cells treated in the same manner as the test cells but without receiving drug. The survival fractions for the different drug concentrations were normalized with respect to the individual controls for each experiment. All experiments were repeated at least twice with triplicate samples for each drug concentration.

Because in many instances the shape of the survival curve obtained for cells in stationary phase differed substantially from that determined for exponentially growing cells, it was impossible to quantify differences in the degree of cell kill by conventional parameters (13). Therefore, we arbitrarily calculated differences in efficacy as the ratio of the survival levels determined at the midrange point of concentrations used for each drug.

Drugs. All drugs (listed in Table 1) were obtained from the Cancer Chemotherapy Evaluation Branch, Division of Cancer Treatment, National Cancer Institute. Drug solutions were always prepared in growth medium immediately before an experiment, and the pH was adjusted if necessary to 7.2 to 7.4. Water-soluble drugs were first dissolved in 0.9% NaCl solution. Lipid soluble drugs were first dissolved in pure ethanol or in 20% ethanol-80% propylene glycol. In some cases (epipodophyllotoxin, methanesulfon-*m*-anisidine, 4′-(acridinylamino), etc.), the drug was dissolved first with the furnished solvent. At the final concentrations used in our experiments, each solvent used alone failed to affect the viability of either exponential or stationary-phase cells.

RESULTS

Proliferation characteristics of LoVo cells inoculated at 3 different cell densities and maintained without refeeding were analyzed. After a lag time of 24 to 36 hr, doubling times were independent of initial inocula and ranged from 34 to 38 hr with a mean value of 36.3 hr. After about 2 doublings (3 to 4 days), the initially higher cell inocula (5 and 7×10^5) entered a phase of stationary growth as defined by no increments in cell number. The plateau segment of the growth curve lasted for about 4 days after which time cell death increased, as reflected by a decrease in cell numbers with a halving time of 103 hr. The LI declined from 33% during exponential growth to 1% just at the time cultures entered stationary phase. However, simultaneously FCM-determined DNA-dependent compartment distributions revealed that about 15% of the cell population had an S-phase DNA content and about 16% of the cells had a G_2-phase DNA content. Stationary phase could not be reverted to exponential growth by: (*a*) replacement of supernatant with fresh medium; (*b*) brief incubation with trypsin and reincubation with the same supernatant; or (*c*) harvesting and transferring the entire cell population to new dishes containing fresh medium. Only when cells were replated in fresh medium at a density lower than 6.45×10^4 cells/sq cm (<1.5×10^6 cells/dish) was logarithmic growth resumed. No significant variations in PE as a function of stage growth (exponential, stationary, or decline) were noted. The fluctuations in PE were similar to those documented for experiments conducted at different times and with different batches of stock cells.

On the basis of these results, survival was compared for exponentially growing LoVo cells (2 to 3 days after subculture) and cells in stationary phase (8 days after subculture). *cis*-Diamminedichloroplatinum was more effective (efficacy ratio,

Table 1

List of antitumor agents used to treat proliferating and nonproliferating cells

Common name	Chemical name	Abbreviation	NSC No.
Adriamycin	14-Hydroxydaunorubicin	ADR	123127
AMSA	Methanesulfon-*m*-anisidide, 4′-(acridinylamino)	AMSA	249992
Anthracenedione	1,4-Dihydro-5,8-bis{{2-[(2-hydroxyethyl)amino]ethyl}amino}9,10-anthracenedione dihydrochloride	DHAQ·Cl	301739
Bleomycin		BLEO	125066
Carmustine	1,3-Bis(2-chloroethyl)-1-nitrosourea	BCNU	409962
	4-[3-(2-Chloroethyl)-3-nitrosoureido]-*cis*-cyclohexanecarboxylic acid	*cis*-Acid	
Cisplatin	*cis*-Diamminedichloroplastinum	*cis*-DDP	119875
Epipodophyllotoxin	4′-Demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-*β*-D-glucopyranoside)	VP-16-213	141540
5-Fluorouracil		5-FU	19893
Hycanthone			142982
Hydroxyurea		HU	32065
Maytansine		MAYT	153858
Methotrexate	Glutamic acid, *N*-{{*p*-{[(2,4-diamino-6-pteridinyl)methyl]methylamino}benzoyl}}	MTX	740
Methyl-GAG	Methylglyoxal bis(guanylhydrazone)	Methyl-GAG	32946
Mitomycin C		Mito C	26980
Rubidazone	Daunorubicin benzoylhydrazone hydrochloride	RUB	164011
Transplatin	*trans*-Diamminedichloroplastinum	*trans*-DDP	
Vinblastine		VBL	49842
Vincristine		VCR	67574
Videsine	Deacetyl vinblastine amide sulfate	VDS	245467

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.
PHIGENIX 1031-03

2.3) on cells in stationary phase than on exponentially growing ones (Chart 1). This effect resulted from the abrogation of the shoulder region of the survival curve, while the slope was maintained essentially intact. Vindesine was also more effective on cells in stationary phase (efficacy ratio of 18.9), but in this case the shape of the survival curve was similar for both cell classes.

Methylglyoxal bis(guanylhydrazone), hycanthone, and vinblastine had a similar low killing effect on both exponentially growing and stationary-phase cells (Chart 2). 1,4-Dihydro-5,8-bis{{{2-[(2-hydroxyethyl)amino]ethyl}amino}}9,10-anthracenedione dihydrochloride (data not shown), mitomycin C, BCNU, and 4-[3-(2-chloroethyl)-3-nitro-ureido]-*cis*-cyclohexanecarboxylic acid (Chart 3) displayed the same powerful effect on both cell classes.

Agents considered to be cell cycle sensitive and to act primarily on cells positioned in the S phase of the cycle (5-fluorouracil, methotrexate, and hydroxyurea) were considerably less lethal on stationary-phase cells (Chart 4; Table 2) than on exponentially growing cells. In fact, methotrexate completely failed to kill cells in the stationary phase of growth.

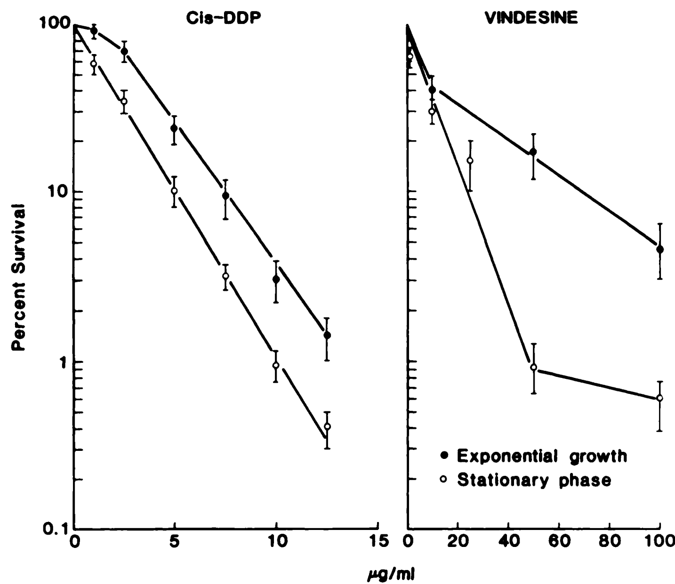

Chart 1. Survival of proliferating and nonproliferating LoVo cells treated with increasing concentrations of *cis*-DDP or VDS for 1 hr. In this, and in subsequent charts, *points* are mean values of at least 2 separate experiments, each with 3 replicates per concentration. *Bars*, S.E.

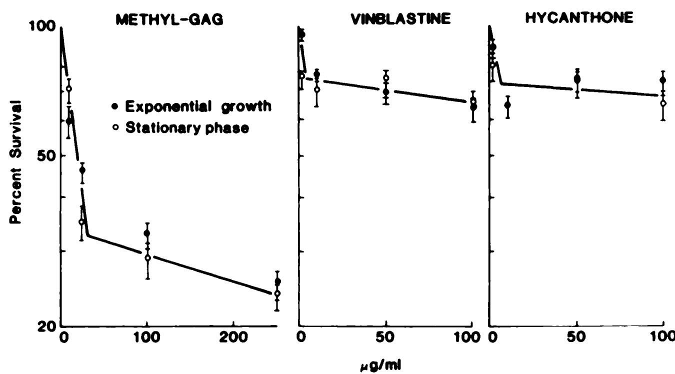

Chart 2. Survival of proliferating and nonproliferating LoVo cells exposed to increasing concentrations of methylglyoxal bis(guanylhydrazone), (*methyl-GAG*), vinblastine, and hycanthone for 1 hr. *Bars*, S.E.

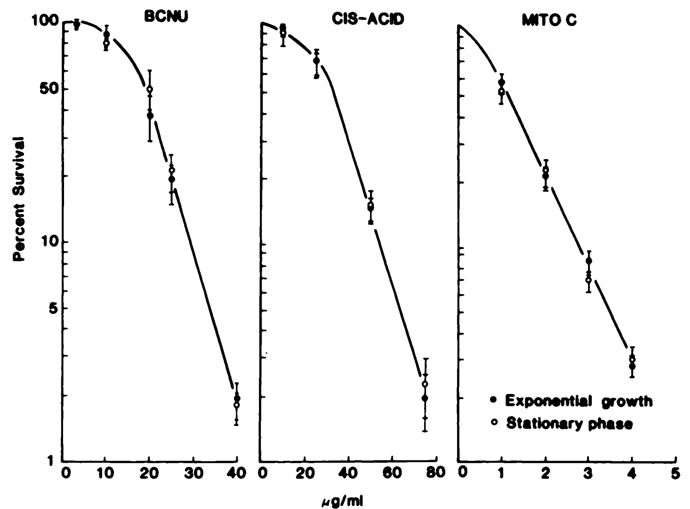

Chart 3. Survival of proliferating and nonproliferating LoVo cells exposed to alkylating agents for 1 hr. *Bars*, S.E. *cis-Acid*, 4-[3-(2-chloroethyl)-3-nitrosoureido]-*cis*-cyclohexanecarboxylic acid; *mito C*, mitomycin C.

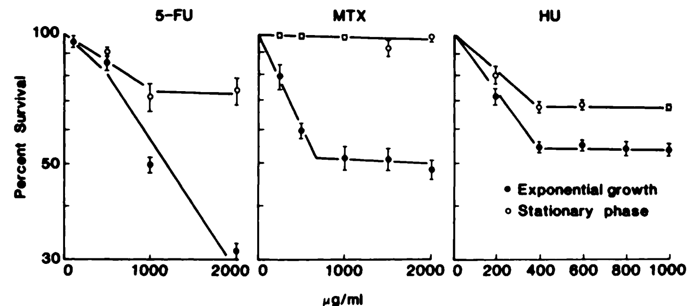

Chart 4. Survival of proliferating and nonproliferating LoVo cells treated for 1 hr with S-phase-sensitive agents. *Bars*, S.E. *5-FU*, 5-fluorouracil; *MTX*, glutamic acid, *N*-{(*p*-{[2,4-diamino-6-pteridinyl)methyl]methylamino}benzoyl}}; *HU*, hydroxyurea.

Table 2

Differential efficacy of antitumor drugs on proliferating and nonproliferating LoVo cells

Drugs	Stationary/exponential[a]
5-FU[b]	1.3
MTX	1.9
HU	1.3
VCR	2.5
MAYT	2.6
VP-16	3.2
ADR	2.5
RUB	3.3
AMSA	3.8
trans-DDP	3.0
BLEO	8.0

[a] Ratio of survivals at midrange dose point.
[b] For definition of abbreviations, see Table 1.

Mitotic inhibitors [vincristine, maytansine, and 4′-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside)] all had a similar type B survival curve (13), and in all instances their efficacy on stationary-phase cells was about 2.5- to 3-fold less than that on exponentially growing cells (Chart 5; Table 2). Similar differences in efficacy between exponential and stationary-phase cells were observed for DNA-intercalating agents such as anthracycline derivatives (adriamycin, daunorubicin benzoylhydrazonehydrochloride) and methanesulfon-*m*-anisidide, 4′-(acridinylamino) (Chart 6). The ineffective *trans* isomer of diamminedichloroplatinum was even less active when

DOCKET ALARM
Find authenticated court documents without watermarks at docketalarm.com.
PHIGENIX 1031-04

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS

Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS

Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS

Sync your system to PACER to automate legal marketing.

WHAT WILL YOU BUILD? | sales@docketalarm.com | 1-866-77-FASTCASE

fastcase® Smarter legal research.