[CANCER RESEARCH 32, 2720–2725, December 1972]

Effects of Cytosine Arabinoside, Daunomycin, Mithramycin, Azacytidine, Adriamycin, and Camptothecin on Mammalian Cell Cycle Traverse[1]

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SUMMARY

Six chemotherapeutic agents were tested for effects on mammalian cell cycle traverse in synchronized cultures of Chinese hamster cells. Cytosine arabinoside was found to inhibit DNA synthesis, as well as to reduce grossly the rate of progression from G_1 into S. At low dosage levels, daunomycin and adriamycin had only a slight effect upon entry of cells into S, but they were almost totally effective in preventing cells from reaching mitosis, and 5-azacytidine greatly inhibited progression into S phase. However, cells already in S at the time azacytidine was added continued to synthesize DNA but were unable to progress to mitosis. Entry into S was hardly affected by mithramycin, while completion of G_2 was partially inhibited, although the sensitivity of G_2 processes was much less pronounced in mithramycin-treated cells than in cells treated with daunomycin or adriamycin. Camptothecin allowed initiation of DNA synthesis, but it effectively prevented cells from progressing to mitosis in the incubation period allotted in the test protocol.

INTRODUCTION

For establishment of truly meaningful chemotherapy regimens, it is essential to pretest agents, not only to determine relative toxicity in host *versus* tumor cells but also to discover possible cell cycle, phase-specific toxic effects and effects on cell cycle progression. In regard to this latter point, the effects on cycle traverse are important, because agents can cause accumulation of cells in specific segments of the cell cycle with increased susceptibility to phase-specific agents. Conversely, agents may be self-limiting if the compound interferes with entry of cells into phases in which cytotoxic effects of the drug are maximal. Effective chemotherapy scheduling must take into account factors such as these, whether the drug in question is administered singly or in combination with other chemotherapeutic agents.

In a previous communication, a simple technique was described for determining the effects of chemotherapeutic agents on mammalian cell cycle traverse (19). The protocol is a functional assay in that it measures the ability of synchronized cells to traverse the cell cycle *in vitro*, based upon determination of the labeled and mitotic fractions in autoradiographs prepared from cultures that received thymidine-^{3}H after the test agent was added in either G_1 or S. In the *in vitro* test system, results were obtained that were comparable to those obtained in published *in vivo* studies, demonstrating the predictive value of the *in vitro* test protocol to *in vivo* chemotherapy trials. In the present report, the test protocol was used to examine effects on cycle traverse of 6 additional agents found to be of value in chemotherapy.

MATERIALS AND METHODS

A line of Chinese hamster cells (line CHO, obtained from Dr. T. T. Puck) was maintained free of pleuropneumonia-like organisms in F-10 medium supplemented with 10% calf and 5% fetal calf sera, penicillin, and streptomycin. Cultures were prepared in a reversible state of G_1 arrest by growth in isoleucine-deficient medium (22). Autoradiographs from cells labeled with thymidine-^{3}H were prepared as described previously (22). All experiments were carried out in 25-ml microspinner flasks (Bellco Biological Glassware, Vineland, N. J.), as described previously.

Thymidine-methyl-^{3}H (6 Ci/mmole) was purchased from Schwarz/Mann, Orangeburg, N. Y. 1-β-D-Arabinofuranosylcytosine (cytosine arabinoside), (NSC 63878) was a gift from the Upjohn Company, Kalamazoo, Mich. Daunomycin (NSC 82151), mithramycin (NSC 24559), 5-azacytidine (NSC 102816), adriamycin (NSC 123127), and sodium camptothecin (NSC 100880) all were obtained through Drug Research and Development, Chemotherapy, National Cancer Institute.

RATIONALE

The rationale for the design of the chemotherapeutic test protocol has been described in detail elsewhere (19). That protocol has been modified slightly, and the amended protocol is shown in Table 1. In this report and in the following paper (21), DNA-synthetic capacity and S phase are arbitrarily defined in terms of the ability of cells to incorporate thymidine-^{3}H and to appear labeled in an autoradiograph. Selection of this method of measurement is based upon technical ease and qualitative nature of results (*i.e.*, labeled or unlabeled cells). As discussed in the accompanying paper (21), there is no universally accepted biochemical definition of initiation of S phase, due primarily to difficulties in deciding semantically what DNA synthesis entails and in obtaining

[1] This study was supported by Contract NIH-CR-(71)-56 from Chemotherapy, National Cancer Institute, NIH, under interagency agreement with the United States Atomic Energy Commission.

Received May 23, 1972; accepted September 5, 1972.

Table 1

Modified protocol for examination of effects of chemotherapeutic agents on cycle traverse with the use of cells maintained in isoleucine-deficient medium for 30 hr

See also Ref. 19.

0 sample

a. Drug added at $t = -1$ hr; all cells in G_1 arrest
b. Isoleucine (to 4×10^{-5} M) and thymidine-^{3}H (to 0.06 μCi/ml) added at $t = 0$; cells still in G_1 but capable of traverse, dependent upon drug action
c. Colcemid (to 0.2 μg/ml) added at $t = 9$ hr (no cell has yet reached M)
d. Autoradiograph prepared at $t = 18$ hr

Control 0

a. Isoleucine (to 4×10^{-5} M) and thymidine-^{3}H (to 0.06 μCi/ml) added at $t = 0$; all cells in G_1 arrest resume cycle traverse
b. Colcemid (to 0.2 μg/ml) added at $t = 9$ hr (no cell has yet reached M)
c. Autoradiograph prepared at $t = 18$ hr

6-hr sample

a. Isoleucine (to 4×10^{-5} M) added at $t = 0$; all cells in G_1 arrest resume cycle traverse
b. Drug added at $t = 6$ hr (25 to 30% of cells in S; remainder in G_1; none as far as G_2)
c. Thymidine-^{3}H (to 0.06 μCi/ml) added at $t = 7$ hr (cell population in G_1 and S; distribution determined by drug used; no cell completed S even in absence of drug effect)
d. Colcemid (to 0.2 μg/ml) added at $t = 9$ hr (no cells yet reached M)
e. Autoradiograph prepared at $t = 18$ hr

Control 7

a. Isoleucine (to 4×10^{-5} M) added at $t = 0$; all cells in G_1 arrest resume cycle tranverse
b. Thymidine-^{3}H (to 2 μCi/ml) added at $t = 6.75$ hr (35 to 40% of cells in S, remainder in G_1; no cell has completed S)
c. Autoradiograph prepared at $t = 7$ hr (40 to 50% of cells in S; remainder in G_1; no cell has completed S)

sufficiently precise measurements of parameters involved in genome replication. Our interpretations are based upon the assumption that thymidine-^{3}H autoradiography represents a valid measure of DNA synthesis. However, no techniques are currently available for extremely precise delineation of the G_1-S boundary.

Cells in G_1 arrest as the result of prolonged growth in isoleucine-deficient medium (22) were given the test agent and, 1 hr later (defined as $t = 0$), isoleucine was added to reinitiate cycle traverse, along with the thymidine-^{3}H, to label cells synthesizing DNA. As a control, G_1-arrested cells were given isoleucine and thymidine-^{3}H at Time 0 (Control 0). Both cultures received Colcemid at $t = 9$ hr to prevent any cell in the population from reentering G_1, and autoradiographs were prepared at $t = 18$ hr for determination of labeled and mitotic cell fractions. If the agent prevents entry into S, there will be neither labeled nor mitotic cells. If the agent allows initiation of DNA synthesis but prevents completion of G_2, the population will consist of labeled, nonmitotic cells. In the absence of effects, labeled and mitotic effects will be equivalent in both cultures.

An additional culture was released from G_1 arrest by the addition of isoleucine at $t = 0$ and, at $t = 6$ hr (~25% of cells in S, the remainder in G_1), the test agent was added. Thymidine-^{3}H and Colcemid were added at 7 and 9 hr, respectively, and the labeled and mitotic cell fractions were calculated from an autoradiograph prepared at $t = 18$ hr. A culture of arrested cells received isoleucine at $t = 0$ and received high levels of thymidine-^{3}H during the period 6.75 to 7 hr, at which time an autoradiograph was prepared that indicated the fraction in S at 7 hr (Control 7). If the agent fails to affect cycle traverse, labeled and mitotic fractions will be equivalent to values in the Control 0 culture. If labeled and mitotic fractions are equivalent to values in the Control 7 sample, the agent specifically inhibits entry into S, since only cells in S at the time of drug addition continue cycle traverse. If the labeled and mitotic fractions are 0, the agent is capable of preventing initiation of genome replication. For a detailed discussion of these and other classes of response obtainable with the test protocol, see the paper of Tobey (19).

RESULTS AND DISCUSSION

Cytosine arabinoside is an agent with a pronounced antineoplastic effect (18). Results obtained with cytosine arabinoside in the test protocol are shown in Table 2. In contrast to the drug-free control, there were no cells in mitosis and few labeled (*i.e.*, DNA-synthesizing) cells in cultures initially receiving drug while the cells were in G_1 arrest. That is, cytosine arabinoside grossly reduced initiation of DNA synthesis, adjudged from an autoradiographic analysis of cells labeled with thymidine-^{3}H. From the cultures first receiving drug at $t = 6$ hr, it may be concluded that cells already in S at the time cytosine arabinoside was added ceased synthesis of DNA within 1 hr (*i.e.*, little label incorporation from 7 hr onward when thymidine-^{3}H is added to the culture), and only a few cells traversed to mitosis. Although cytosine arabinoside grossly reduced the number of cells entering S phase, over prolonged periods of time the labeled fraction slowly rose (as an example, see Ref. 21), indicating that a few cells continued to enter S phase even in the presence of 5 or 10 μg of the drug per ml. As is shown in the following paper (21), when cytosine arabinoside was washed out of cells after a 1-hr incubation period, nearly all of the cells immediately entered S phase and were capable of continuing cycle traverse for at least 2 rounds of division. These results suggest that cells remaining in G_1 were spared from the cytotoxic effects of cytosine arabinoside. The results obtained indicate that the rate of progression of synchronized CHO cells into S phase in cytosine arabinoside-treated cultures is both time and concentration dependent, with movement from G_1 into S drastically reduced.

A variety of techniques have been used to examine the effects of cytosine arabinoside on the initiation of DNA synthesis in mammalian cells, but conflicting results have been obtained. Some investigators have suggested inhibition of entry into S (1, 9, 13), while others have concluded that cells initially in G_2, M, or G_1 either enter S and are killed (11) or enter S but remain viable (3, 15). Much of the confusion that arises is attributable to the difficulty in obtaining definitive data on cell cycle kinetics, as well as to problems in defining S phase. Consequently, in the accompanying paper (21), a new technique is described that allows a highly detailed analysis of initiation of genome replication, and consideration is given to the semantic problems involved in defining S phase. The protocol uses synchronized CHO cells grown *in vitro*, and the

Table 2
Effects of cytosine arabinoside on cell cycle traverse

Time drug was added (hr)	Effects with the following concentrations of cytosine arabinoside							
	10 μg/ml		5 μg/ml		2 μg/ml		0	
	LF[a]	MF	LF	MF	LF	MF	LF	MF
0[b]	0.044	0	0.088	0	0.116	0	0.884	0.446
6[c]	0.016	0	0.040	0	0.744	0.432		
7 (control)[d]							0.546	0

[a] LF, fraction labeled with thymidine-^{3}H (measured autoradiographically); MF, fraction of cells in mitosis. All mitoses scored were labeled with thymidine-^{3}H, since the original culture contained a nearly pure population of cells in G_1.

[b] Cells maintained for 30 hr in an isoleucine-deficient medium were given the appropriate concentration of drug at $t = -1$ hr and, at $t = 0$, each culture was given thymidine-^{3}H to a final concentration of 0.06 μCi/ml, along with isoleucine to a final concentration of 4×10^{-5} M. The drug-free control received both thymidine-^{3}H and isoleucine at $t = 0$. All cultures received Colcemid to 0.2 μg/ml at $t = 9$ hr. Autoradiographs were prepared at $t = 18$ hr. Each culture contained 25 ml at 200,000 cells/ml.

[c] Cells maintained for 30 hr in isoleucine-deficient medium were given isoleucine to a final concentration of 4×10^{-5} M at $t = 0$. The cultures received the appropriate concentration of drug at $t = 6$ hr and were given thymidine-^{3}H to a final concentration of 0.06 μCi/ml at 7 hr. Colcemid was added to all cultures at 9 hr to a final concentration of 0.2 μg/ml. Autoradiographs were prepared at $t = 18$ hr.

[d] The 7-hr control culture received isoleucine to a final concentration of 4×10^{-5} M at $t = 0$; then thymidine-^{3}H was added to a concentration of 2 μCi/ml at $t = 6.75$ hr. At $t = 7$ hr, the sample was prepared for autoradiography.

Table 3
Effects of daunomycin on cell cycle traverse
The protocol and abbreviations are described in Table 1. Abbreviations are as in Table 2.

Time drug was added (hr)	Effects with the following concentrations of daunomycin							
	4 μg/ml		1 μg/ml		0.5 μg/ml		0	
	LF	MF	LF	MF	LF	MF	LF	MF
0	0.420	0	0.806	0	0.830	0.002	0.928	0.468
6	0.816	0.002	0.894	0.002	0.888	0.004		
7 (control)							0.420	0

parameters determined include autoradiographic and cell enumeration, as well as DNA distribution patterns obtained with the Los Alamos flow microfluorometer. In agreement with the results shown in Table 2, the results obtained (21) indicate that cytosine arabinoside grossly reduces the transition of cells from G_1 to an active S phase and causes accumulation of cells in the vicinity of the G_1-S boundary. Ramifications of these findings to chemotherapy are considered in the accompanying paper (21).

Daunomycin is an effective antileukemic agent (25). Upon examination with the *in vitro* test system, the results shown in Table 3 were obtained. All of the treated cultures are essentially devoid of mitotic cells, whereas the fraction that synthesizes DNA is uniformly high in all cultures, except that treated with the antibiotic (4 μg/ml) during G_1 arrest. These results suggest that daunomycin does not greatly affect the G_1 to S transition but is extremely effective in preventing cells from reaching mitosis. It is not clear whether the inability to reach mitosis arises from a specific effect upon G_2 processes or whether it is due to the synthesis of a grossly defective genome in the presence of the drug, which is incapable of transcribing appropriate messengers for completion of G_2 operations. *In vitro* studies of daunomycin effects in mouse L-cells (4) and human HeLa cells (7) and *in vivo* studies of drug effects on peripheral blood leukocytes in human leukemias (25) basically were all in agreement. Daunomycin appears to affect both DNA and RNA synthesis but, at low doses, initiation and completion of mitosis are extremely sensitive. The highly labeled fraction in most of the cultures shown in Table 3 reflects the ability of cells to initiate DNA synthesis and to continue synthesizing DNA, although these data give no indication on the relative rate of DNA synthesis or the degree of completeness of genome replication in the presence of daunomycin. Thus, results reported here for effects of daunomycin agree well with results obtained *in vivo* or *in vitro* in other laboratories.

Mithramycin is an antibiotic that inhibits growth of rodent tumors and is clinically effective in the treatment of embryonal cell carcinoma of the testis, hypernephroma, and glioblastoma multiforme (5, 12). *In vitro* studies in HeLa cells (17) and *in vitro* and *in vivo* studies of the murine ascites tumor 6C3HED (26) showed that mithramycin primarily inhibits RNA synthesis, with very little effect upon DNA synthesis. Consequently, we should expect the test system to yield results that indicate a high percentage of labeled cells in all samples and, at higher drug concentrations, a gross

inhibition of mitosis indicative of the effects of agents primarily affecting RNA synthesis [for example, see dactinomycin (19)]. Such is indeed the case in Table 4, pointing up the necessity for RNA-associated processes in the G_2 phase of the cell cycle [see review of biochemistry of G_2 by Tobey *et al.* (23)].

5-Azacytidine is a compound that, in early preclinical trials, has been shown to be active against murine L1210 leukemia (24). When azacytidine was examined by the test protocol, the results shown in Table 5 were obtained. In cultures that received azacytidine while the cells were in G_1 arrest, initiation of DNA synthesis was drastically inhibited at all concentrations studied. Results from cultures that received azacytidine at 6 hr indicate that cells in S phase at the time of drug addition continue to incorporate thymidine into DNA for at least 1 hr but that few cells enter S and commence synthesizing DNA. Those cells initially in S phase at the time of drug addition have difficulty in reaching mitosis, but the data in Table 5 do not reveal whether these cells completed genome replication. This compound has a primary effect upon initiation of genome replication and a secondary effect upon completion of interphase.

Adriamycin is a close structural analog of daunomycin but is more effective against murine L1210 leukemia than is daunomycin (16). As expected from the structural similarity, results obtained in the test system (Table 6) are very similar to results obtained with daunomycin (Table 3). Initiation of genome replication is not grossly inhibited by the drug, whereas completion of mitosis is extremely sensitive to the effects of adriamycin. Kim and Kim (14) have shown that HeLa cell viability is most sharply reduced in cells treated during S phase; thus, it is probable that cells never reach mitosis because they are unable to complete DNA synthesis and G_2. Progression to mitosis is much more strongly inhibited in cells treated with either daunomycin (Table 3) or adriamycin (Table 6) than in mithramycin-treated cells (Table 4), perhaps suggesting a different mechanism of inhibition of G_2 processes by the 2 classes of compounds. Although both mithramycin and daunomycin (and presumably also adriamycin) inhibit RNA synthesis (7, 17), the exquisite sensitivity to daunomycin or adriamycin may result from an effect on a process other than RNA synthesis. For example, daunomycin forms a variety of complexes with DNA (6), and one could speculate that the structure of DNA complexed

Table 4

Effects of mithramycin on cell cycle traverse

The protocol is as described in Table 1. Abbreviations are as in Table 2.

Time drug was added (hr)	Effects with the following concentrations of mithramycin							
	5 μg/ml		1 μg/ml		0.5 μg/ml		0	
	LF	MF	LF	MF	LF	MF	LF	MF
0	0.788	0	0.920	0.172	0.924	0.470	0.938	0.558
6	0.952	0.176	0.914	0.318	0.904	0.306		
7 (control)							0.494	0

Table 5

Effects of 5-azacytidine on cell cycle traverse

The protocol is as described in Table 1. Abbreviations are as in Table 2.

Time drug was added (hr)	Effects with the following concentrations of azacytidine							
	10 μg/ml		5 μg/ml		1 μg/ml		0	
	LF	MF	LF	MF	LF	MF	LF	MF
0	0.016	0	0.014	0	0.070	0	0.830	0.466
6	0.508	0.004	0.628	0.008	0.666	0.152		
7 (control							0.510	0

Table 6

Effects of adriamycin on cell cycle traverse

The protocol is as described in Table 1. Abgreviations are as in Table 2.

Time drug was added (hr)	Effects with the following concentrations of adriamycin							
	2 μg/ml		0.8 μg/ml		0.4 μg/ml		0	
	LF	MF	LF	MF	LF	MF	LF	MF
0	0.808	0.010	0.792	0.004	0.888	0.010	0.874	0.370
6	0.890	0	0.906	0.004	0.898	0.050		
7 (control)							0.498	0

Table 7
Effects of sodium camptothecin on cell cycle traverse
The protocol is as described in Table 1. Abbreviations are as in Table 2.

Time drug was added (hr)	Effects with the following concentrations of sodium camptothecin									
	2 μg/ml		1 μg/ml		0.5 μg/ml		0.05 μg/ml		0	
	LF	MF	LF	MF	LF	MF	LF	MF	LF	MF
0	0.660	0	0.822	0.002	0.824	0	0.822	0.281	0.820	0.470
6	0.740	0	0.822	0	0.844	0.004	0.839	0.322		
7 (control)									0.638	0

with drug was sufficiently deformed that normal chromosome condensation could not occur. However, at least 1 report of mithramycin binding to DNA has appeared (17). Data on the relative degree of binding of agents to DNA are required, as well as information on structural properties of DNA-drug complexes, before one can seriously consider models of the type described above.

The antibiotic camptothecin has antitumor and antileukemic activity (8). This compound affects synthesis of both RNA and DNA in mammalian cells (2, 10). Examination of the effects of this compound with the test protocol yielded the results shown in Table 7. Camptothecin had little or no effect on initiation of genome replication but, instead, had a pronounced effect upon completion of interphase. As is shown in the accompanying paper (21) with a new technique that used flow microfluorometry, although most cells in the population have initiated DNA synthesis (*i.e.*, are labeled) at the time of preparation of the autoradiographs at $t = 18$ hr, few cells have completed synthesis of a full complement of DNA. These results are in contrast to those obtained with another antitumor compound, bleomycin, which allows initiation and synthesis of a full complement of DNA at a normal rate (19–21).

ACKNOWLEDGMENTS

The excellent technical assistance of Mrs. Susan G. Carpenter and Mrs. Phyllis C. Sanders is gratefully acknowledged.

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