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AMPHOTERICIN B: A BIOLOGICAL RESPONSE MODIFIER IN TARGETING AGAINST THE SALVAGE PATHWAYS*

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Abstract—Dipyridamole, a nucleoside transport inhibitor which blocks the rescue effect of exogenous nucleosides, is a compound of interest for use in combination with antimetabolites of *de novo* purine and pyrimidine biosynthesis. This study has provided evidence that the dipyridamole inhibitory effect on nucleoside incorporation varied markedly during the course of cell growth in culture. Human colon cancer HT-29 cells in lag and log phases were highly sensitive to the blocking action of dipyridamole on thymidine incorporation with IC_{50} values of 0.06 and 0.07 μM respectively. In contrast, stationary phase cells were comparatively insensitive to dipyridamole with an IC_{50} of 46 μM. Amphotericin B restored the sensitivity of stationary phase cells to dipyridamole, lowering the IC_{50} value for thymidine incorporation to 0.03 μM. A similar pattern was observed for cytidine incorporation. Amphotericin B also enhanced the growth inhibitory action of dipyridamole in stationary phase cells. The combination of amphotericin B and dipyridamole should be potentially useful in cancer chemotherapy.

We suggested that the biochemical reason for the lack of effectiveness of antimetabolites of purine and pyrimidine *de novo* biosynthesis in producing lasting remissions is the presence in cancer cells of high enzymic capacity for salvage of preformed nucleosides and bases [1, 2]. The cytotoxic effectiveness of antimetabolites should be improved by inhibitors of the transport of nucleosides and nucleobases because this would block the effectiveness of salvage [1, 2]. Since no specific inhibitors of the salvage enzymes were available, the effect of dipyridamole, a potent inhibitor of the transport of nucleosides and nucleobases, was studied, and cytotoxic synergism was obtained by combining acivicin and dipyridamole in rapidly growing hepatoma 3924A cells [1, 2]. Our investigations were also the first to show that dipyridamole alone is cytotoxic to cancer cells [2].

Recently we observed that the sensitivity to the action of dipyridamole in inhibiting transport is altered profoundly with the growth phase of the cancer cells. In hepatoma cells in lag and log phases there is a higher sensitivity to inhibition of transport by dipyridamole; however, in the stationary phase the cells lose sensitivity to dipyridamole [3]. To sensitize plateau phase cancer cells to the action of dipyridamole, we examined a number of compounds which act on the cell surface and determined that amphotericin B, an antibiotic and anti-fungal agent, acts as a biological response modifier and renders stationary hepatoma cells sensitive to dipyridamole [3].

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This paper shows that amphotericin B can be used as a biological response modifier by making plateau phase human colon carcinoma cells (HT-29) sensitive to the action of dipyridamole.

METHODS

Materials. Acivicin was obtained from the Upjohn Co., Kalamazoo, MI. Amphotericin B, dipyridamole and nucleosides were purchased from the Sigma Chemical Co., St. Louis, MO. [U-^{14}C]Cytidine and organic counting scintillant were purchased from the Amersham Corp., Arlington Heights, IL, and [2-^{14}C]thymidine from New England Nuclear, Boston, MA.

Cell culture. The human colon cancer cell line HT-29 was obtained from Dr. R. H. Shoemaker (National Cancer Institute). This cell line was established from a primary colon adenocarcinoma obtained from a female patient and shows an epithelial-like appearance in culture [4]. HT-29 cells were grown in RPMI 1640 medium (Grand Island Biological Co., Grand Island, NY), supplemented with 10% fetal calf serum, penicillin, 100 units/ml, and streptomycin, 100 μg/ml. The cells were incubated at 37° in a 5% CO_2–95% air humidified atmosphere. For subculture, the cells were dispersed with 0.025% trypsin plus 1 mM EDTA in phosphate-buffered saline (containing, in g/liter, KCl, 0.2; KH_2PO_4, 0.2; NaCl, 8.0; and Na_2HPO_4, 1.15) at 37° for 15 min. After centrifugation, the supernatant fraction was removed, and the cells were resuspended in fresh medium and seeded in culture flasks. For nucleoside incorporation assay and growth inhibition studies, cells were seeded in 24-well plates at a density of 10^4 cells/cm^2 (2×10^4 cells per ml per well).

Nucleoside incorporation assay. The ^{14}C-labeled nucleoside stock solution was made up to 2 μCi/ml (50 mCi/mmol) before use. The assay of the nucleoside incorporation was performed by first remov-

ing the spent medium and then washing the wells with 1 ml of cold phosphate-buffered saline after which 1 ml of serum-free medium was pipetted into each of the wells. Then 20 μl of amphotericin B or dipyridamole and ^{14}C-labeled nucleoside were added. In all experiments, amphotericin B was added 5 min before dipyridamole and the latter was added 5 min before the ^{14}C-labeled nucleoside. Duplicate samples were used for each concentration tested. After a 60-min incubation, the wells were analyzed for the incorporation of the ^{14}C-labeled nucleoside into trichloroacetic acid-insoluble material. The medium was removed, and the wells were rinsed with ice-cold phosphate-buffered saline. Then 0.2 ml of NaOH (0.2 N) was added to dissolve the cells followed by 2 ml of ice-cold 10% trichloroacetic acid. After 30 min, the contents of the wells were filtered on Whatman glass fiber discs (934-AH, 2.4 cm, Whatman, Inc., Clifton, NJ) held in place by a Millipore microanalysis filtration apparatus. The filters were washed with 10 ml of ice-cold 5% trichloroacetic acid and 1 ml of 95% ethanol. The dried discs were then counted in 10 ml of organic counting scintillant fluid by a Beckman liquid scintillation LS 3801 system.

Growth inhibition studies. Exponentially growing cells were seeded in 24-well plates in medium containing 10% dialyzed fetal bovine serum. For growth inhibition studies of cells in various growth phases, two drug exposure protocols were used, the first of which was for cells in lag and log phases. Under this condition, the drugs were added 6 hr after seeding and maintained in medium for 90 hr. The second protocol utilized was to study the growth inhibition of stationary phase cells. At 96 hr after seeding, the spent medium was removed, and cells were washed with phosphate-buffered saline and replenished with fresh medium containing dialyzed serum. Then the drugs were added for a 24-hr exposure period. Triplicate samples were used for each concentration. Cells were harvested by trypsin plus EDTA treatment as described and counted by a Coulter Counter. Cell numbers from the treated and untreated groups were compared after separately subtracting the initial cell count determined before the addition of drugs.

RESULTS

Insensitivity of stationary phase cells to dipyridamole. The growth curve for human colon cancer HT-29 cells is shown in Fig. 1. When the cells were seeded at 10^4 cells/cm^2, the lag, log and stationary phases occurred at 0–24, 24–96 and 96–144 hr respectively. The cell densities at mid log phase (48 hr) and late stationary phase (144 hr) were 3.3×10^4 and 2.8×10^5 cells/cm^2. Thymidine incorporation was much higher in log phase cells than in stationary phase cells with a peak at 48 hr.

As shown in Fig. 2, inhibition of thymidine incor-

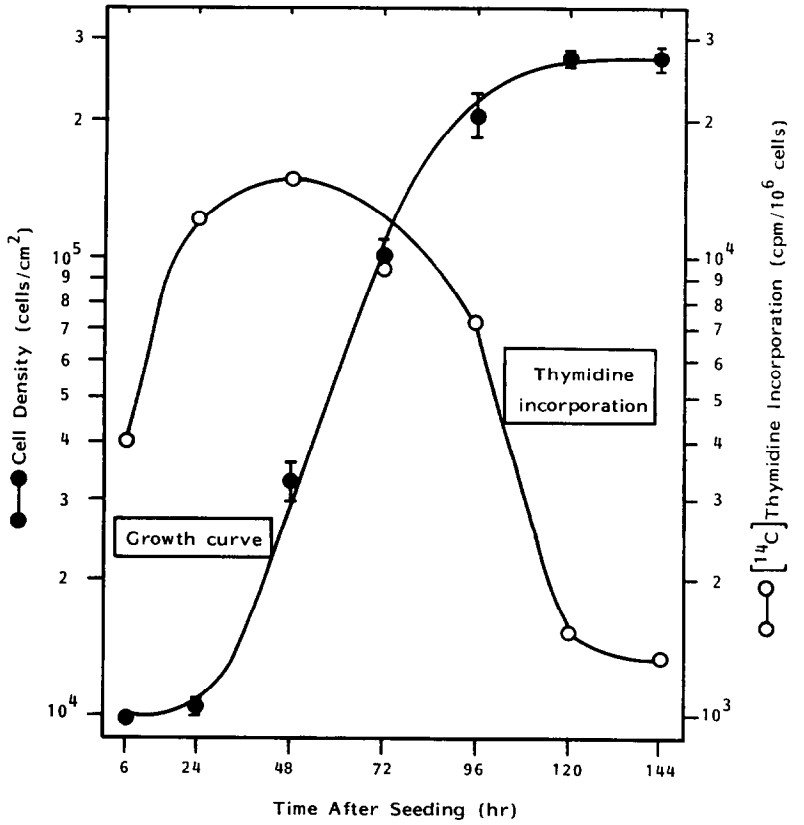

Fig. 1. Growth curve for human colon cancer HT-29 cells and thymidine incorporation at different times after seeding. Cells were seeded at a density of 10^4 cells/cm^2. The procedure for the cell count and thymidine incorporation assay was as described in Methods. Points for cell count are means ± SE of triplicate samples. Points for thymidine incorporation are means of duplicate determinations.

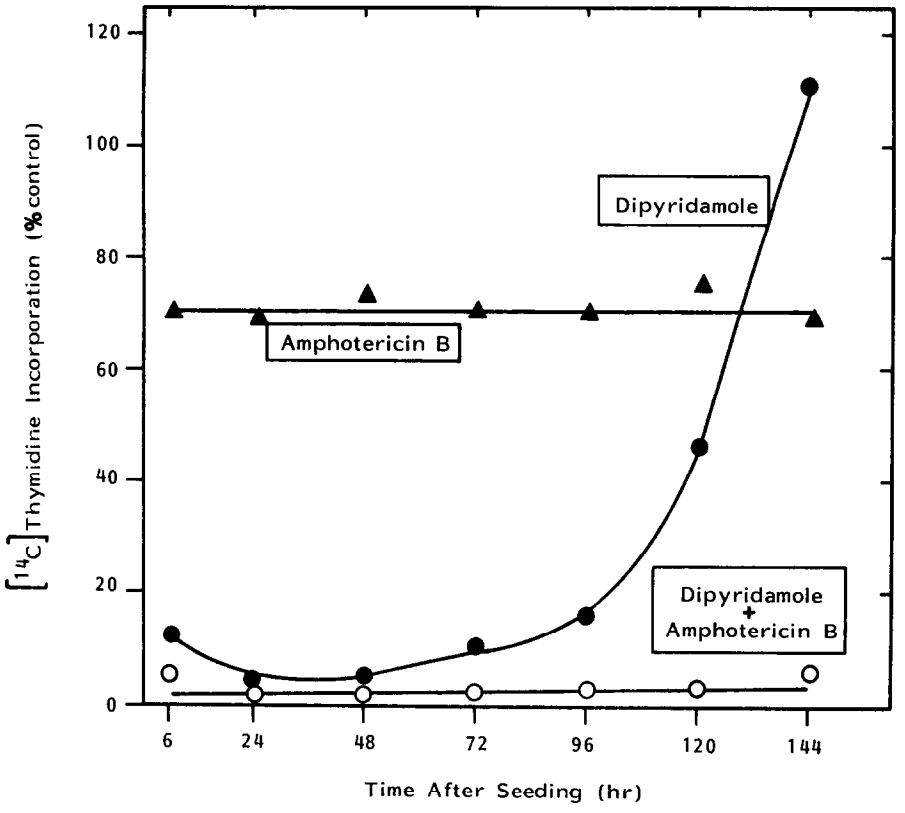

Fig. 2. Inhibition of thymidine incorporation by dipyridamole and amphotericin B in various growth phases of HT-29 cells. Amphotericin B (10 μM) was added 5 min prior to dipyridamole (10 μM). The procedure for thymidine incorporation was as described in Methods. Points are means of duplicate determinations.

poration by dipyridamole changed with growth phase. Cells in lag and log phases were highly sensitive to dipyridamole (10 μM) where 90–95% of thymidine incorporation was inhibited. In contrast, late stationary cells (144 hr) were insensitive to dipyridamole action, displaying no inhibition of thymidine incorporation. In lag and log phase cells, for the inhibition by dipyridamole of thymidine incorporation the IC_{50} values were 0.06 and 0.07 μM, respectively, whereas in stationary phase the IC_{50} was 46 μM. The inhibitory action of dipyridamole on cytidine incorporation revealed similar changes in the various growth phases (Table 1). Trypan blue dye exclusion test demonstrated that in the untreated late stationary phase cells only 1–2% of the cell population was stained.

Table 1. Effect of amphotericin B on the inhibitory activity of dipyridamole on nucleoside incorporation in HT-29 cells

Cell phase*	Treatment†	Dipyridamole IC_{50}‡ (μM)	
		Incorporation	
	Dipyridamole	Cytidine	Thymidine
Lag	—	0.02	0.06
	+Amphotericin B	0.01	0.02
Log	—	0.02	0.07
	+Amphotericin B	0.01	0.03
Stationary	—	5.60	46.00
	+Amphotericin B	0.04	0.03

*Lag phase cells, 6 hr after seeding; log phase cells, 48 hr after seeding; stationary cells, 144 hr after seeding.

†In combination, cells were treated with amphotericin B (10 μM) for 5 min before dipyridamole was added and then incubated in the presence of both drugs. After the addition of graded concentrations of dipyridamole (5 min), ^{14}C-labeled nucleoside was added, and cells were incubated at 37° for 60 min.

‡Radioactivity in trichloroacetic acid-insoluble fractions was measured. The IC_{50} values were determined from log dose–response curves where graded concentrations of dipyridamole (0.01 to 0.16 μM) were added to lag and log phase cells and (5 to 40 μM) to stationary phase cells.

The amphotericin B preparation consisted of 45% amphotericin B, 35% sodium deoxycholate, and sodium phosphate. The amphotericin B concentrations utilized in this investigation were corrected for the fact that the solubilized stock solution contained 45% of this drug. When added alone, amphotericin B (10 μM) inhibited thymidine incorporation by 20–35% during the various cell phases (Fig. 2). We also examined the effect of sodium deoxycholate on thymidine incorporation using a sodium deoxycholate concentration of 7.18 μg/ml which equalled the content of amphotericin B in a 10 μM solution. The sodium deoxycholate showed no effect on thymidine incorporation and failed to restore the sensitivity to dipyridamole in stationary phase cells (data not shown).

Table 2. Dose–response effect of amphotericin B in rendering plateau phase HT-29 sensitive to the inhibition of thymidine incorporation

	Dipyridamole (μM)	Amphotericin B (μM)	Thymidine incorporation (% control)
Control	—	—	100
	10	—	100
	—	10.00	69
	10	0.61	86
	10	1.25	38
	10	2.50	22
	10	5.00	13
	10	10.00	10

Cells at 144 hr after seeding were used for the assay. Amphotericin B was added 5 min prior to dipyridamole.

Dose–response effect of amphotericin B in restoring sensitivity to dipyridamole in stationary phase colon carcinoma cells. The cells were pretreated with amphotericin B for 5 min followed by the addition of dipyridamole and 5 min later the labeled nucleoside was added for a 60-min incubation as described in Methods. In the presence of amphotericin B (10 μM), stationary phase HT-29 cells became highly sensitive to dipyridamole with more than 90% of thymidine incorporation inhibited (Fig. 2). In combination with amphotericin B, the IC_{50} values of dipyridamole for cytidine and thymidine incorporation in stationary phase cells were 0.04 and 0.03 μM, respectively, which closely approximated the IC_{50} values for lag or log phase cells (Table 1). Thus, amphotericin B rendered stationary phase cells as sensitive to dipyridamole action as lag and log phase cells were. Lower concentrations of amphotericin B (to 1.25 μM) were also effective in restoring the sensitivity of stationary phase cells to dipyridamole (Table 2).

Using higher concentrations for amphotericin B alone, only minor inhibitory effects on thymidine incorporation were observed in both log and stationary phase cells with IC_{50} values of 19 and 25 μM respectively (not shown).

Synergistic action of amphotericin B on growth inhibition by dipyridamole in stationary phase cells. As shown in Table 3, both dipyridamole and amphotericin B alone exhibited growth inhibition of HT-29 cells. The IC_{50} of dipyridamole for lag to log phase cells was 20 μM which closely agreed with the IC_{50} value observed for the rat hepatoma 3924A cells [2]. Stationary phase cells were less sensitive to dipyridamole with an IC_{50} of 81 μM. However, the effects of amphotericin B on growth in log and stationary phase cells were similar, the IC_{50} values being 46 and 42 μM.

In combination, amphotericin B (10 μM), in a concentration without growth inhibitory effect in itself, resulted in a synergistic cytoxicity by dipyridamole in stationary phase cells. In contrast, in lag and log phase cells only summation was found (Table 4).

Effect of dipyridamole and amphotericin B on rescue action of nucleosides in stationary phase cells. As shown in Table 5, acivicin inhibited the growth of stationary phase HT-29 cells. The addition of a nucleoside mixture (cytidine, deoxycytidine and guanosine) protected the cells from acivicin. Dipyridamole alone partially blocked this protection, whereas dipyridamole in combination with amphotericin B eliminated the nucleoside rescue effect resulting in a cessation of HT-29 cell growth.

DISCUSSION

We suggested that the cytotoxic effectiveness of antimetabolites should be improved by dipyridamole, an inhibitor of the transport of nucleosides and nucleobases, and demonstrated that such synergism is obtained by combining acivicin and dipyridamole [1, 2]. These investigations were the first to show that dipyridamole kills cancer cells [2]. These novel observations and our suggestion that the combina-

Table 3. Growth inhibition of various growth phases of HT-29 cells by dipyridamole and amphotericin B

Growth phase	Drug exposure time (hr)	Dipyridamole IC_{50} (μM)	Amphotericin B IC_{50} (μM)
Lag to log	6–96	20	46
Stationary	96–120	81	42

The IC_{50} values were determined from log dose–response curves where graded concentrations of dipyridamole (lag to log phase, 12–50 μM; stationary phase, 27–75 μM) or amphotericin B (5–100 μM) were added at the designated time after cell seeding and maintained for the exposure period as indicated. Triplicate wells were used for each concentration.

Table 4. Amphotericin B enhanced growth inhibition by dipyridamole in stationary phase HT-29 cells

Growth phase	Drug exposure time (hr)	Cell count (% control, untreated cells) Dipyridamole (a)	Amphotercin B (b)	Dipyridamole + Amphotericin B Predicted (c)	Measured (d)
Lag or log	6–96	37	91	34	27
Stationary	96–120	72	100	72	0*

After seeding cells, dipyridamole or amphotericin B was added and maintained in medium for the indicated periods. Drug concentrations used were amphotericin B, 10 μM; dipyridamole, 25 μM, for lag to log phase cells, and 75 μM for stationary phase cells respectively. Amphotericin B was added 5 min before dipyridamole. Cells were harvested by trypsin plus EDTA and counted by a Coulter Counter as described in Methods. Results are means of three wells for each point and expressed as percent of untreated control; $c = (a \times b)/100$.

*Significant synergism: $d < 0.7c$.

tion of dipyridamole and antimetabolites should have direct clinical application were followed by reports on cytotoxic synergism of dipyridamole with methotrexate [5–7], 5-fluorouracil [8], *N*-phosphonacetyl-L-aspartate [9, 10], arabinosyl cytosine [11], deoxyadenosine and deoxycoformycin [12]. It was also shown that dipyridamole alone inhibits the growth of human glioblastoma and neuroblastoma cells [13].

The present study confirmed and extended our original observations that the sensitivity to dipyridamole markedly changes during the course of growth in culture [3]. Human colon cancer HT-29 cells in lag and log phase were highly sensitive to dipyridamole, whereas cells in the stationary phase were insensitive. On the basis of IC_{50} values for thymidine incorporation, the difference in sensitivity between log (0.07 μM) and stationary (46.0 μM) phase cells was 766-fold (Table 1). Stationary phase cells were also less sensitive to the growth inhibitory action of dipyridamole. The insensitivity of stationary phase cells to dipyridamole is particularly important for solid tumors where there is a small growth fraction which is less sensitive to the antimetabolite and other anticancer drugs [14].

In lag and log phase cells, the presence of amphotericin B yielded only slight changes in the IC_{50} values. However, the combination with amphotericin B made stationary phase cells 1533-fold more sensitive to dipyridamole (46.0 to 0.03 μM) (Table 1). These results indicated that amphotericin B exhibited a selective action on stationary phase cells rendering them sensitive to the inhibitory action of dipyridamole.

Amphotericin B is an anti-fungal antibiotic which binds to sterols in the eukaryotic cell membrane and increases membrane permeability. As reported, amphotericin B inhibits thymidine incorporation in L cells [15]. In our study in HT-29 cells, amphotericin B alone at higher concentrations inhibited thymidine incorporation; however, no difference in sensitivity between log phase and stationary phase cells was observed (Table 3).

The growth inhibition study in stationary phase cells also showed that dipyridamole failed to block the nucleoside rescue against acivicin; however, by using a combination of amphotericin B and dipyridamole, the rescue effect was eliminated completely (Table 5).

Amphotericin alone exhibits no established antitumor activity; however, it has been shown to potentiate the effect of various chemotherapeutic agents by causing increased drug uptake [15–19] and has been

Table 5. Effects of acivicin, amphotericin and dipyridamole on HT-29 cell growth in stationary phase

Treatment*	Cell count (% control)
Amphotericin B	118
Acivicin	18
Acivicin + nucleosides†	150
Acivicin + nucleosides + dipyridamole	58
Acivicin + nucleosides + dipyridamole + amphotericin B	0‡

Assay for growth inhibition was as described in Methods. Triplicate samples were determined for each experimental group.

*Drugs were added at 96 hr after seeding and maintained for 24 hr. Concentrations of drugs used were: acivicin (100 μM), dipyridamole (10 μM), and amphotericin B (10 μM).

†Nucleosides consisted of cytidine (100 μM), deoxycytidine (100 μM), and guanosine (200 μM).

‡Synergism.

used in combination with antitumor drugs in clinical trials [20]. In addition, amphotericin B has been shown to induce drug sensitivity in previously drug-resistant cells [20]. Our earlier work showed that amphotericin B rendered plateau phase rat hepatoma cells sensitive to the action of dipyridamole [3]. The presented new data show the applicability of these observations to human colon cancer cells and extend the significance of this approach to combination drug treatment with acivicin, amphotericin B and dipyridamole. Thus, our novel results point to the feasibility of using amphotericin B as a *biological response modifier* in combination with dipyridamole. Both amphotericin B and dipyridamole are licensed drugs; therefore, this combination may be potentially useful in the antimetabolite chemotherapy of neoplastic diseases.

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