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Concentration-Dependent Effects of Foscarnet on the Cell Cycle

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The mechanism of toxicity of foscarnet was studied by monitoring its effects on the cell cycle of exponentially growing, semisynchronous human embryo cells in culture. The effects of foscarnet on the cell cycle were dependent on the concentration of drug used. At 1 mM, cell division was reduced by 50%, whereas the cell flow was mainly reduced in the G_2 phase of the cell cycle, leading to an increase in the proportion of G_2+M cells. The minor reduction of thymidine incorporation in S phase cells provided additional evidence that 1 mM foscarnet did not specifically inhibit DNA synthesis. Cell division was greatly reduced at 2.5 mM foscarnet, and the G_2 phase was markedly affected, whereas S cell flow was less reduced. S cell flow was 10% per h and thymidine incorporation was 25% that of control cells, while a block in the G_2+M phase was evident. On the other hand, at a concentration of 5 mM foscarnet, the cell flow was greatly reduced in the G_1 and S phases, with less reduction of G_2 cell flow and cells accumulated in the S phase. The effects of foscarnet on the cell cycle were more pronounced with increasing times up to 72 h, which could not be explained by the slow penetration of foscarnet which required only 4 to 8 h to achieve constant levels. At 2.5 and 5 mM foscarnet, there was the additional effect of the cell membranes becoming more leaky as a result of foscarnet toxicity which might contribute to the toxic effects of the drug at high concentrations. When foscarnet was removed from the medium, the effects on the cell cycle were rapidly reversed, in the time needed for foscarnet to diffuse out from the cells, which indicates the reversible nature of the toxic effects of foscarnet.

Foscarnet (trisodium phosphonoformate) has been shown to be an effective inhibitor of the multiplication of several DNA viruses and retroviruses (see reference 8 for a review). The antiviral effect of foscarnet, a pyrophosphate analog, is mediated by an inhibition of DNA synthesis by interference with the pyrophosphate-binding site on the DNA polymerase (9, 10). At higher concentrations of foscarnet, the eucaryotic DNA polymerase α was inhibited, although a variable inhibition was seen, depending on the amount of enzyme present in the assay (6).

In uninfected cells, foscarnet inhibited DNA synthesis more efficiently than RNA or protein synthesis, and these effects were reversible (12). Cells treated with ≤5 mM foscarnet for one cell generation time were accumulated in the S phase of the cell cycle, and DNA synthesis was inhibited in proportion to the inhibition of growth, indicating that the toxic mechanism of foscarnet was due to inhibition of DNA synthesis (13). The low toxicity reported for foscarnet on resting cells (8), which have a low percentage of S-phase cells, gives further evidence that cellular DNA synthesis is the main target for foscarnet toxicity. In this paper, we report on a study of the effects of foscarnet on the cell cycle of semisynchronous human cells. The levels of foscarnet in the cells were monitored during the experiment, and the effect of foscarnet on membrane permeability was determined.

MATERIALS AND METHODS

Cells, media, and radiolabeled compounds. A finite human embryo fibroblast cell line, Flow 1000, obtained from Flow Laboratories, Irvine, United Kingdom, was used in passages 15 to 20. Minimal essential medium with Earle salt, fetal calf serum, and trypsin-EDTA solution were purchased from Gibco Bio-Cult, Paisley, United Kingdom. [^{14}C]foscarnet (specific activity, 430 MBq/μmol) was synthesized by L. Gawell, Astra Läkemedel AB (4). RNase type III-A and pepsin were from Sigma Chemical Co., St. Louis, Mo. [*methyl*-^{3}H]thymidine (specific activity, 740 MBq/μmol) was from New England Nuclear Corp., Boston, Mass.

Cell growth. Stock cultures of Flow 1000 cells were grown at 37°C in plastic tissue culture flasks (75-cm^2 growth area; Nunc, Roskilde, Denmark) in a medium containing 10% fetal calf serum, penicillin (120 μg/ml), and streptomycin (100 μg/ml). The cultures were subcultivated every 3 to 4 days with a split ratio of 1:2. Stock cultures, with 90 to 95% of the cells in the G_1 phase, were seeded at a density of 10^6 cells in each dish of a six-well multidish (35-mm diameter; Costar, Cambridge, Mass.) and left at 37°C for 16 h in a 5% CO_2–humidified air mixture to allow for cells to start entering the S and G_2 phases of the cell cycle. Foscarnet was then added to the desired final concentrations from a 100-mM stock solution made in distilled water (pH 7.2). At the indicated times, duplicate dishes were trypsinized to obtain single cells, which were checked by phase-contrast microscopy. The cell suspension was then diluted in particle-free buffer (Baker Chemicals, Deventer, The Netherlands) and counted in an electronic cell counter (Analysinstrument, Stockholm, Sweden).

Analysis of cellular DNA content. Duplicate dishes were washed three times with Tris-EDTA buffer (pH 7.5; 0.1 M Tris, 0.07 M NaCl, 0.005 M EDTA). Cells were then trypsinized, pelleted by centrifugation, and stored in absolute ethanol at −20°C until they were prepared for flow cytometry. Individual cell nuclei were prepared by being washed in Tris-EDTA buffer followed by RNase treatment and digestion by pepsin. The DNA was then stained with ethidium bromide, and the DNA content of the cell nuclei was determined with a flow cytometer (ICP 11; Phywe, Federal Republic of Germany). The excitation wavelength was 455 to 490 nm, and the emission wavelength was 590 to 630 nm. The results of each measurement were sorted and stored with a 256-channel analyzer (5, 14). The proportions

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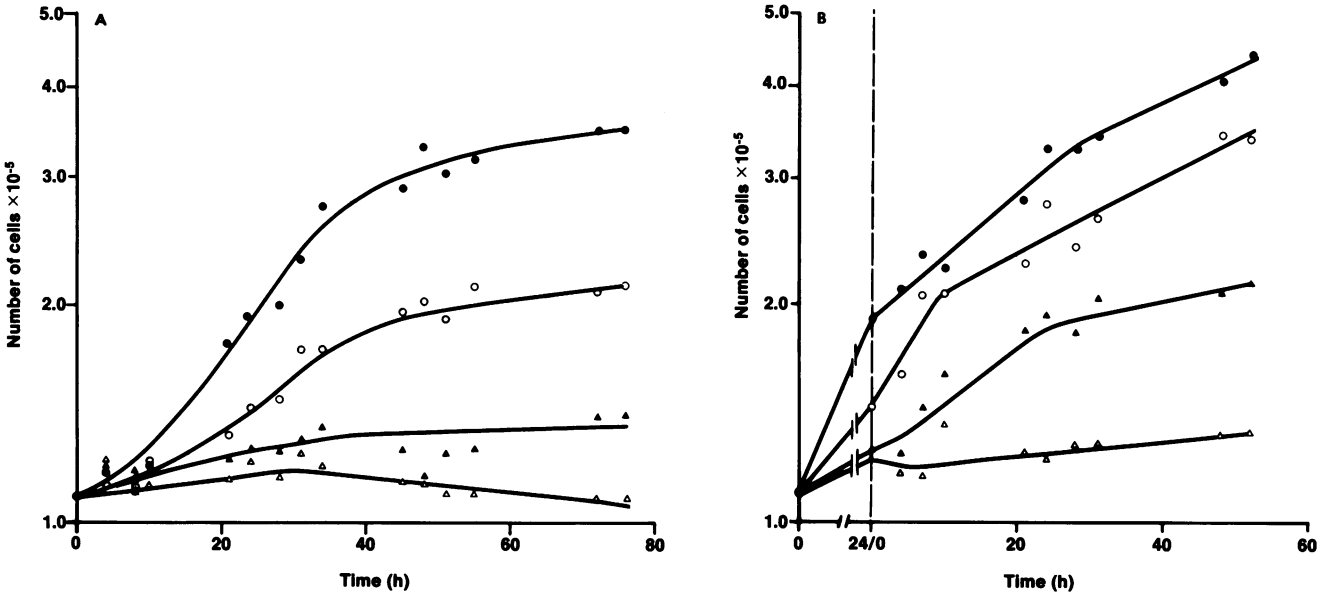

FIG. 1. Effect of foscarnet on cell division and regrowth after drug removal. Foscarnet was added to the growth medium of exponentially growing human embryo cells at 0 h, and cell division was monitored for 72 h. At the indicated times, duplicate dishes were trypsinized and cell numbers were counted (A). Regrowth was studied by incubation with 1 to 5 mM foscarnet for 24 h followed by a change to a new growth medium. Cell division in the absence of foscarnet was monitored for 48 h (B). Symbols: ●, untreated cells; ○, 1 mM foscarnet; ▲, 2.5 mM foscarnet; △, 5 mM foscarnet.

of cells in different cell cycle phases, G_1, S, and G_2+M, were determined from the areas of the histograms, assuming a Gaussian distribution of the G_1 and G_2+M peaks and attributing the remaining part of the DNA histogram to the S phase cells.

Calculation of cell flow. The cell flow through each cell cycle phase was calculated by measuring the changes in cell number and the changes in the proportions of cells in the G_1, S, and G_2 + M phases during certain time periods (see legend to Fig. 3) after the addition of different concentrations of foscarnet (10a). Because the number of cells in each cell cycle phase varied with drug concentration and time after the addition of the drug, the data are presented as the relative cell flow. This is equal to the sum of the number of cells entering and leaving each cell cycle phase during 1 h divided by 2, relative to the total number of cells present in each phase.

Incorporation of radioactive thymidine into S phase cells. The rate of DNA synthesis was estimated by adding [^{3}H]thymidine (specific activity, 740 MBq/μmol or 75 kBq/ml) for 30 min to cells treated for different times with foscarnet. The acid-insoluble material, obtained after washing five times with 2 ml of phosphate-buffered saline and five times with 2 ml of ice-cold 5% trichloroacetic acid, was solubilized in 1 ml of 0.5 M NaOH at 80°C for 20 min. The alkali-soluble material obtained was neutralized with 100 μl of 5 M HCl before being counted in a scintillator.

Levels of foscarnet in the cells. Different concentrations of [^{14}C]foscarnet were added to exponentially growing cells in the growth medium. At the indicated times, duplicate dishes were washed five times with 2 ml of ice-cold phosphate-buffered saline. The cell monolayers were then solubilized with 0.5 M NaOH and counted in a scintillator as described above. Experiments to study the release of foscarnet were done by treating cells with [^{14}C]foscarnet for 24 h followed by one wash with prewarmed phosphate-buffered saline before the addition of new growth medium. Cell-associated radioactivity was measured after digestion with 0.5 M NaOH at 80°C for 20 min.

Changes in the uptake of foscarnet. At different times during incubation with 1 to 5 mM unlabeled foscarnet, [^{14}C]foscarnet was added (10^6 cpm per dish) to duplicate dishes as a 30-min pulse to measure the rate of uptake. After the 30-min pulse, cell monolayers were rapidly washed five times with 2 ml of ice-cold phosphate-buffered saline to remove extracellular drug. The remaining, cell-associated radioactivity was then measured by scintillation counting after the cells were solubilized with 0.5 M NaOH as described above.

RESULTS

Effects of foscarnet on cell division and the effect of removing the drug. The effect of the continuous presence of foscarnet on cell division and the effect of removing foscarnet from the growth medium after 24 h of incubation are shown in Fig. 1A and 1B. Cell division was monitored for 72 h, including 40 h of exponential growth. Cell division was reduced by 50% at 1 mM foscarnet, whereas only a small increase in cell number was noted at a concentration of 2.5 mM (Fig. 1A). Even at 5 mM, an initial increase in cell number was seen, but after 30 h there was a significant cell detachment from the culture dishes which resulted in a net decrease in cell number. At concentrations less than 1 mM, no inhibition of growth was detected, and at 10 mM cell growth was completely arrested.

To study the reversibility of drug toxicity, cells were treated with 1 to 5 mM foscarnet for 24 h, followed by a change to fresh growth medium and reincubation for 48 h (Fig. 1B). Cells recovering from 1 mM foscarnet showed more rapid cell division than untreated cells, whereas with 2.5 mM cell division was as rapid as for untreated cells. The recovery of cells treated with 5 mM foscarnet was low, because the increase in cell number was less than 20% of that of control cells. This was due both to a slow cell division and

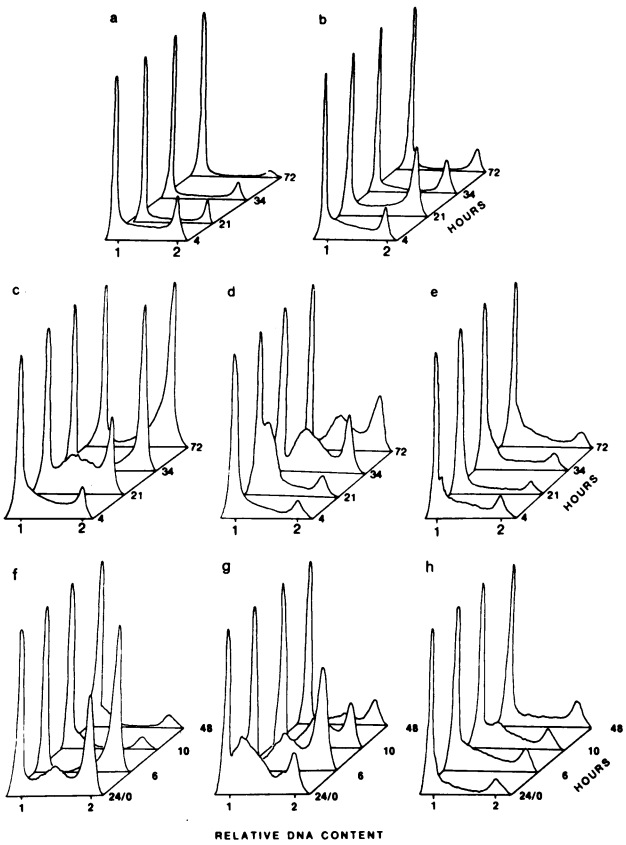

FIG. 2. Concentration-dependent cell cycle accumulation after treatment with foscarnet. DNA histograms represent untreated cells (a) and cells treated with 1 mM (b), 2.5 mM (c), 5 mM (d), or 10 mM (e) foscarnet for different time periods. After 24 h of incubation, foscarnet (2.5 mM [f], 5 mM [g], or 10 mM [h]) was removed by washing with prewarmed phosphate-buffered saline. New growth medium was added, and the DNA content was recorded at the times indicated in the figure. A relative DNA content of 1 represents G_1 cells, and a content of 2 is equal to G_2+M cells, whereas S phase cells have a DNA content between 1 and 2.

to a cell loss per h of 2 to 3% of total cell number after removal of the drug.

Concentration-dependent accumulation in different cell cycle phases by foscarnet. Untreated cells showed a high proportion of cells in the S and G_2 phases of the cell cycle at 4 h, and upon prolonged incubation cells gradually accumulated in the G_1 phase (Fig. 2a). Treatment with 1 mM foscarnet decreased the number of late S-phase cells 4 h later (Fig. 2b). At 21 h, there was a significant increase in the proportion of G_2+M cells which later decreased, but at 72 h the percentage of G_2+M cells was still two times that of the untreated cells. In the presence of 2.5 mM foscarnet, a high proportion of cells was found in the mid-S phase after 21 h which at 34 h was changed to a late S and G_2+M block (Fig. 2c). At 5 mM foscarnet, a high proportion of early-S-phase cells was observed which at 34 h was shifted to a mid-S and G_2+M block (Fig. 2d). When cells were treated with 10 mM foscarnet, they were found at the border between the G_1 and S phases for all samples analyzed at 4 to 72 h (Fig. 2e).

When foscarnet was removed after 24 h of incubation, the mid-S and G_2+M accumulation at 2.5 mM was shifted to a high proportion of cells in G_2+M 6 h later (Fig. 2f). However, after 10 h, more than 80% of the cells were found in the G_1 phase, which correlates with the increase in cell division within 10 h after removal of the drug (Fig. 1B). The high proportion of early-S-phase cells found after treatment with 5 mM foscarnet were, 6 h after removal, found in the mid-S and G_2+M phases (Fig. 2g). However, a significant percentage of cells was still in the late S and G_2+M phases even after 48 h without the drug. The high proportion of cells at the G_1-S border in the presence of 10 mM foscarnet was shifted to early S phase, but 48 h after removal there was still a higher proportion of S and G_2+M cells than untreated cells (Fig. 2h).

Relative cell flow in the presence of foscarnet and after removing the drug. Data from several determinations of cell number and DNA content have been combined to show the effect of foscarnet on the cell cycle kinetics of human embryo cells. The relative cell flow describes how active the cells are in passing through each specific cell cycle phase. For example, a relative cell flow of 10% per h in G_1 means that the sum of the cells that have entered and left G_1 in 1 h is equal to 10% of the total number of cells in G_1.

Untreated cells had a relative cell flow through the G_1 phase of less than 10% and through G_2+M of 20%, whereas S phase flow increased from 10 to 28% during 60 h of incubation, indicating stimulation of S cell flow during cell growth. After 60 h, a decrease in S phase flow was noted (data not shown). At 1 mM foscarnet, the cell flow through the G_1 and S phases was reduced only by about 20%, whereas a 50% reduction in G_2+M flow was noted at 24 h (Fig. 3A). This is in line with the accumulation of G_2+M cells found after 21 h (Fig. 2b) and the reduction of cell division by 50% (Fig. 1A).

At 2.5 or 5 mM foscarnet, the cell flow through the G_1 phase was close to 0 24 h after addition of the drug. After 24 h, at 2.5 but not at 5 mM foscarnet, there was a marked decrease in the G_2+M cell flow, which correlates with the high proportion of G_2+M cells found at 34 to 72 h with 2.5 mM foscarnet (Fig. 2c). At both 2.5 and 5 mM foscarnet, the S cell flow was gradually reduced, and after 60 h, cell flow was undetectable.

In Fig. 3B, the effect on the cell flow of removing 1 to 5 mM foscarnet after 24 h is shown for the first 21 h after removal. The removal of 1, 2.5, or 5 mM foscarnet stimulated the cell flow in all cell cycle phases within 6 h in a manner similar to the response of untreated cells to the change of growth medium (Fig. 3B). However, the stimulation of cells in G_2+M after 2.5 mM and of G_1 cells after 5 mM foscarnet was delayed about 3 h. After the removal of 1 or 2.5 mm foscarnet, a threefold increase in G_1 cell flow was noted (Fig. 3B), together with a twofold increase in S cell flow after the removal of 1 mM foscarnet or a threefold increase in S cell flow after the removal of 2.5 mM. Removing 1 to 5 mM foscarnet increased cell flow in the G_2+M phase but to a lesser extent. After 21 h, the cell flow gradually returned to that of the untreated cells (data not shown).

Effect of foscarnet on the incorporation of thymidine into S phase cells. DNA synthesis was measured as the amount of [^{3}H]thymidine incorporated into acid-precipitable material and related to the number of cells capable of DNA synthesis, because, in the presence of foscarnet, the proportion of cells in S phase varies with both time and drug concentration. [^{3}H]thymidine incorporation was maximal at 36 h for both untreated cells and cells treated with foscarnet (Fig. 4). A reduction by 10 to 20% of [^{3}H]thymidine incorporation at 1 mM foscarnet between 24 and 48 h was observed, which was significantly less than the 50% reduction in cell division at 1

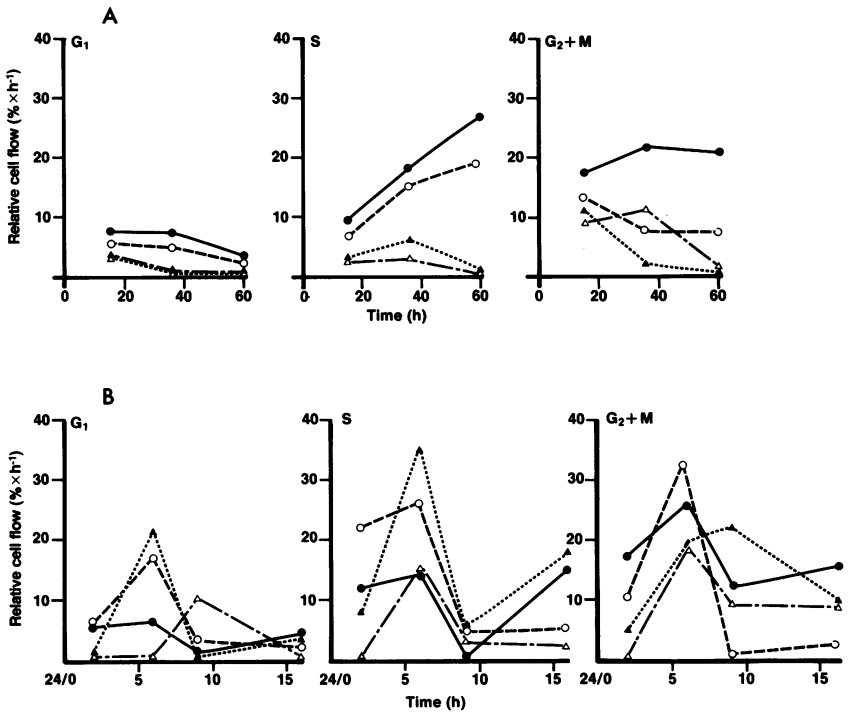

FIG. 3. Relative cell flow rate through different phases of the cell cycle. (A) Flow rate in the presence of foscarnet. Data shown are the mean rates during 4 to 24, 24 to 45, and 45 to 72 h. (B) Flow rate after removing foscarnet after 24 h of treatment. Data shown are the mean rates during 0 to 4, 4 to 7, 7 to 10, and 10 to 21 h after drug removal. Symbols: ●, untreated cells; ○, 1 mM foscarnet; ▲, 2.5 mM foscarnet; △, 5 mM foscarnet.

mM (Fig. 1A). Even at 2.5 mM foscarnet, the inhibition of [^{3}H]thymidine incorporation was far from complete: 0.10 cpm per S phase cell in 30 min compared with 0.37 cpm per S phase cell in 30 min for untreated cells. However, after 48 h with 2.5 or 5 mM foscarnet or after 72 h with 1 mM foscarnet, DNA synthesis as measured by [^{3}H]thymidine incorporation was efficiently inhibited.

Levels of foscarnet in cells. The amount of foscarnet associated with cells increased initially, and a constant level was found at 4 to 8 h after addition of the drug (Fig. 5). This uptake kinetics shows that the cell cycle effects observed after this initial time period can be considered proportional to the extracellular concentration. However, the uptake of foscarnet was 8 to 16 times slower than that of orthophosphate, which is of similar molecular weight and charge (data not shown).

The release of foscarnet from cells after change of medium apparently followed the same kinetics as for the uptake (Fig. 5). This indicates that the effects of 1 to 2.5 mM foscarnet are

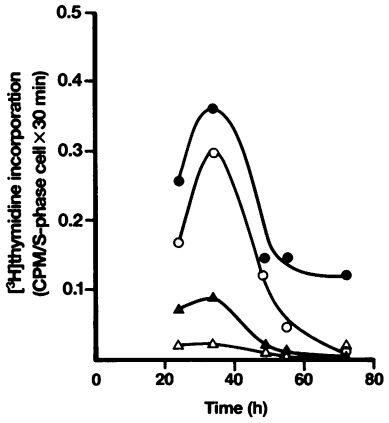

FIG. 4. [^{3}H]thymidine incorporation in S phase cells in the presence of foscarnet. At the indicated times, [^{3}H]thymidine (6 MBq/ml) was added to the incubation medium containing different concentrations of foscarnet. The acid-insoluble radioactivity formed during 30 min is presented per S phase cell which was determined by flow cytometry. Symbols: ●, untreated cells; ○, 1 mM foscarnet; ▲, 2.5 mM foscarnet; △, 5 mM foscarnet.

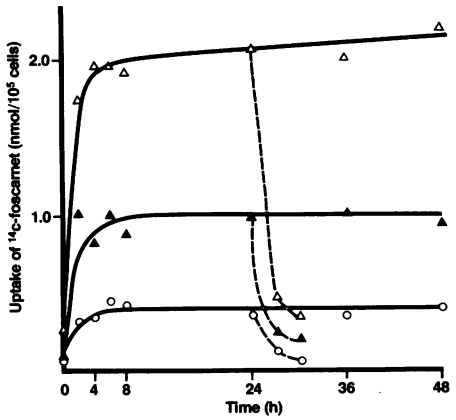

FIG. 5. Time course of the uptake and release of [^{14}C]foscarnet. Uptake (solid line) of foscarnet was followed by treating exponentially growing cultures with 1-mM (○), 2.5-mM (▲), and 5-mM (△) concentrations. Release (broken line) was studied after drug treatment for 24 h. A total of 500 cpm was equivalent to 1 nmol of foscarnet.

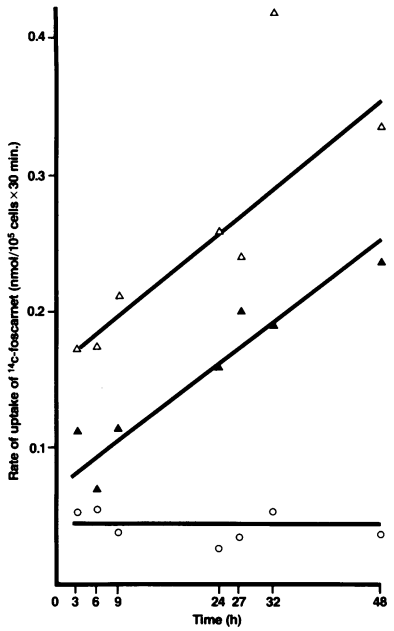

FIG. 6. Foscarnet uptake at different times during drug treatment. [^{14}C]foscarnet was added as 30-min pulses at different times after the addition of 1 to 5 mM drug to monitor differences in the rate of uptake. Symbols: ○, 1 mM foscarnet; ▲, 2.5 mM foscarnet; △, 5 mM foscarnet.

rapidly reversed, because cell division and cell flow were stimulated within 6 h after extracellular drug was removed (Fig. 1B and 3B), the amount of time required for foscarnet to leave the cells.

Different rate of foscarnet uptake as a result of drug toxicity. Because toxicity is often associated with permeability changes, we studied how the rate of foscarnet uptake changes during 48 h of treatment with 1 to 5 mM foscarnet. This was performed by adding [^{14}C]foscarnet as 30-min pulses at different times after unlabeled foscarnet was added to the cultures. When cells were treated with 1 mM foscarnet, the rate of uptake was almost constant thoughout the 48-h incubation period (Fig. 6). In contrast, at 2.5 or 5 mM foscarnet, there was a twofold increase in the rate of foscarnet uptake, indicating that membrane leakage was a result of the toxicity of foscarnet at concentrations of 2.5 mM or higher.

DISCUSSION

This study showed that the effect of foscarnet on the cell cycle was dependent on the concentration of drug used. At 1 or 2.5 mM foscarnet, at which cell growth was reduced by 50 and 90%, respectively, the main effect of foscarnet was a reduction of the flow of G_2 cells, whereas at ≥5 mM, S phase flow and [^{3}H]thymidine incorporation were strongly inhibited.

The effect of foscarnet on cellular DNA synthesis and the S phase correlated with earlier observations (11–13) and was analogous to the antiviral mechanism of action of foscarnet (8). On the other hand, the inhibition in the G_2 phase indicated that processes active in the G_2 phase also were affected by foscarnet. The mechanism of G_2 inhibition cannot be determined from the present experiments. However, with respect to the DNA-specific effect at higher concentrations, one possible explanation is that nonreplicative DNA synthesis in the G_2 phase might be more affected than is replicative DNA synthesis in the S phase.

For structurally different inhibitors of DNA synthesis, concentration-dependent effects on the cell cycle, similar to those seen for foscarnet, were demonstrated. Treatment with MICs of 1 to 2 μM aphidicolin, an inhibitor of DNA polymerase α, or inorganic, hexavalent chromium, results in a G_2 accumulation while, by increasing the concentration, a block in the S phase is induced (1, 3).

In addition, if the increased permeability seen at ≥2.5 mM foscarnet is involved in the shift from the G_2 to the S phase, inhibition remains to be demonstrated.

Inhibition of progression in all cell cycle phases was reversed within the time required for foscarnet to diffuse out from the cells, indicating a reversible mechanism of inhibition of the drug.

ACKNOWLEDGMENTS

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