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An inhibitor of glycinamide ribonucleotide formyltransferase is selectively cytotoxic to cells that lack a functional G1 checkpoint

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Abstract *Purpose*: We studied the effects of purine depletion on the cell cycle using a specific inhibitor of *de novo* purine biosynthesis, AG2034, an inhibitor of glycinamide ribonucleotide formyltransferase (GARFT). *Methods*: Cytotoxicity was determined by clonogenic assays, and cell cycle perturbations by flow cytometry. Ribonucleotide pools were measured by anion exchange high-pressure liquid chromatography, and DNA strand-breaks were determined by alkaline elution and by the TUNEL assay. *Results*: When cells were maintained in standard tissue culture medium, which contained 2.2 μ*M* folic acid, AG2034 was cytostatic in all the cell lines tested. Under low-folate conditions (50 n*M* folic acid), AG2034 caused up to 50% cell death in cell lines that possessed a functional G1 checkpoint (A549, MCF-7), but was only cytostatic to the remaining cells, even at very high concentrations (100 μ*M*). In contrast, AG2034 at 10 n*M* or 100 n*M* killed all the cells in cultures of HeLa/S3 or SW480 cells, which lack a functional G1 checkpoint. Flow cytometry studies indicated that in G1 checkpoint-competent cells, AG2034 caused a G1 arrest. Those cells (up to 50%) that were already in S phase died, but the cells that were in G1 arrest maintained viability, based upon clonogenic assays, for many days. In G1 checkpoint-deficient cells, no G1 arrest was seen after AG2034 treatment, all cells progressed into S phase, and all cells died. Measurement of DNA strand-breaks, either by alkaline elution or by the dUTP end-labelling technique, indicated no DNA strand-breaks 24 h after AG2034 treatment, indicating that purine nucleotide depletion can trigger the G1 checkpoint in the absence of DNA damage. *Conclusion*: Purine depletion causes slow cell death in cells that have passed the G1 checkpoint, but cytostasis in cells that are arrested at the G1 checkpoint. The GARFT inhibitor, at physiological folate concentrations, thus causes selective cytotoxicity to cells lacking a functional G1 checkpoint.

Key words AG2034 · G1 checkpoint · G1 arrest · GARFT · Ribonucleotide depletion

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Introduction

It is estimated that more than half of human tumors have a mutation or defect in the cell cycle checkpoint that controls progression from G1 to S phase of the cell cycle. These mutations often involve the p53 tumor suppresser protein [15]. Tumors lacking a functional G1 checkpoint are generally relatively refractory to radiotherapy and chemotherapy [7]. Cancer treatment would probably be more generally effective if new agents could be identified that retain effectiveness against $p53^-$ tumor cells. In addition, since cells of normal tissues are presumably $p53^+$, agents that selectively kill checkpoint-defective cells should have a high degree of antitumor selectivity. Bischoff et al. [1] have described the use of a mutant adenovirus that selectively destroys $p53^-$ cells, and this virus is currently in clinical trials as an anticancer agent. We here describe an alternative approach based upon the use of a small-molecule drug that selectively kills p53-deficient cells.

AG2034 (Fig. 1) is a 28 n*M* inhibitor of glycinamide ribonucleotide formyltransferase (GARFT), an enzyme that forms part of the purine *de novo* biosynthetic pathway. AG2034 was produced by structure-based drug design from the GARFT X-ray crystal structure [14], and it is known to bind to the reduced folate cofactor site. It has been shown to have antitumor activity against murine tumors and human tumor xenografts [2]. Previous reports have variously described earlier GARFT inhibitors as being cytostatic or as cytotoxic [11, 12]. We here present evidence that the cell cycle effect and cytotoxicity of AG2034 depend upon whether or not the cell possesses a functional G1 checkpoint.

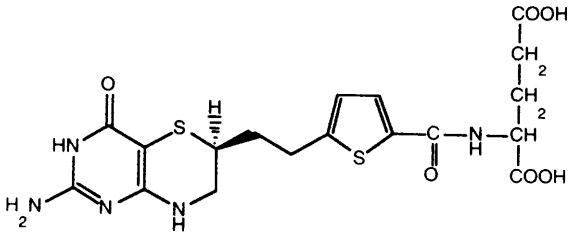

Fig. 1 Chemical structure of AG2034, 4-[2-(2-amino-4-oxo-4,6,7,8-tetrahydro-3H-pyrimidino[5,4-6][1,4]thiazin-6-yl)-(*S*)-ethyl]-2,5-thienoyl-L-glutamic acid

Methods

Cell culture

The HeLa/S3 human cervical carcinoma, MCF-7 human breast cancer, SW480 human colon carcinoma, A549 and H460 human nonsmall-cell lung carcinomas and L1210 murine leukemia cell lines were obtained from the American Type Culture Collection, Rockville, Md. L1210 cells were maintained in RPMI-1640 medium containing 10% dialyzed fetal bovine serum. Other cells were adapted to minimal essential medium (MEM Select-Amine kit, Gibco, Grand Island, NY) containing the desired folic acid concentrations and 10% fetal bovine serum (A549, H460, MCF-7) or charcoal/dextran-dialyzed fetal bovine serum (HeLa/S3, SW480). Before running experiments, cells were maintained in low-folate medium until their growth rate was the same as in normal (2.2 μ*M* folic acid) medium. For clonogenic assays, mid-log-phase cells were trypsinized and placed in 60-mm Petri dishes at 150 cells per dish. Triplicates were used for each point. Cells were left for 4 h to attach and then treated with AG2034 for 2 weeks, at which time visible colonies were obtained in the control plates. Colonies were fixed with ethanol and stained with 1% w/v methylene blue before counting. The cloning efficiency for HeLa/S3, A549 and H460 cells was 90–95%, and for SW480, H460 and MCF-7 cells was about 60%. Cell synchronization was performed as described by O'Connor and Jackman [9], modified to use a single thymidine blockade.

Ribonucleotide assays

Trypsinized cells were counted and centrifuged. Ice-cold 0.7 *M* perchloric acid (0.5 ml) was added to each cell pellet. After centrifugation, supernatants were transferred to microfuge tubes, and neutralized with solid potassium bicarbonate before analysis by anion exchange chromatography [8].

Flow cytometry

Cell cycle analysis was performed using a FACSCalibur fluorescence-activated cell sorter (Becton-Dickinson, San Jose, Calif.). Approximately 1 million cells per test were stained for DNA with propidium iodide, using the Becton-Dickinson TEST PLUS kit.

DNA strand-break assays

Measurement of DNA strand-breaks by the terminal deoxynucleotide transferase-mediated dUTP nick end-labelling technique, known as the TUNEL assay [4], was carried out using the kit available from Boehringer Mannheim Corporation Indianapolis, IN. Alkaline elution assays followed the standard procedure of Kohn et al. [5].

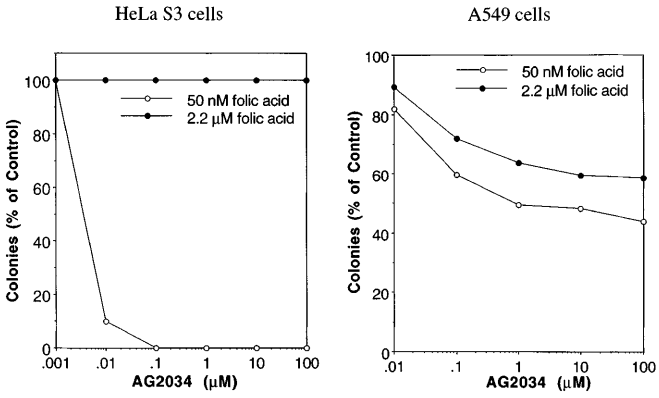

Fig. 2 Effect of folic acid concentration on the cytotoxicity of AG2034 in HeLa/S3 and A549 cells. The cells were plated in 60-mm Petri dishes at 150 cells per dish with MEM containing either 2.2 μ*M* or 50 n*M* folic acid in the presence or absence of AG2034, and counted after 14 days (HeLa/S3) or 21 days (A549)

Results

Our studies indicated that AG2034 was cytostatic to HeLa/S3 cells grown in standard MEM, which contained 2.2 μ*M* folic acid, but when MEM containing 50 n*M* folic acid was used (closer to physiological conditions), AG2034 was highly cytotoxic to HeLa/S3 cells. In contrast, growth in low-folate conditions only slightly increased cytotoxicity to A549 human lung carcinoma cells (Fig. 2) even after exposure for up to 3 weeks. Because of the marked effect of the folate concentration in the culture medium on the response of the cells to AG2034, all the subsequent experiments, described below, were carried out in medium containing 50 n*M* folic acid.

The observation that 0.1 μ*M* AG2034 was completely lethal to HeLa/S3 cells but that a concentration 3 logs higher gave only 50% kill of A549 cells at physiological folate concentrations prompted extension of this study to further cell types (Fig. 3). The SW480 human colon carcinoma cell line responded similarly to HeLa/S3, in this case 10 n*M* AG2034 giving complete cell kill. The human breast carcinoma line, MCF-7, like A549, only suffered about 50% cell kill after prolonged exposure to 100 μ*M* AG2034 (Fig. 3). These results suggested a sharp dichotomy between the response to AG2034 of cells with a functional G1 checkpoint (A549, MCF-7), where only a minor degree of cytotoxicity was seen, even at high concentrations, and cells lacking a functional G1 checkpoint (HeLa/S3, SW480) which were highly sensitive to AG2034-induced cytotoxicity. Measurements of purine ribonucleotide pools by anion exchange high-pressure liquid chromatography in HeLa/S3, MCF-7 and A549 cells showed marked decreases in all these lines (Table 1). Thus the inability of AG2034 to kill A549 and MCF-7 cells was not a result of a lack of effect on *de novo* purine biosynthesis.

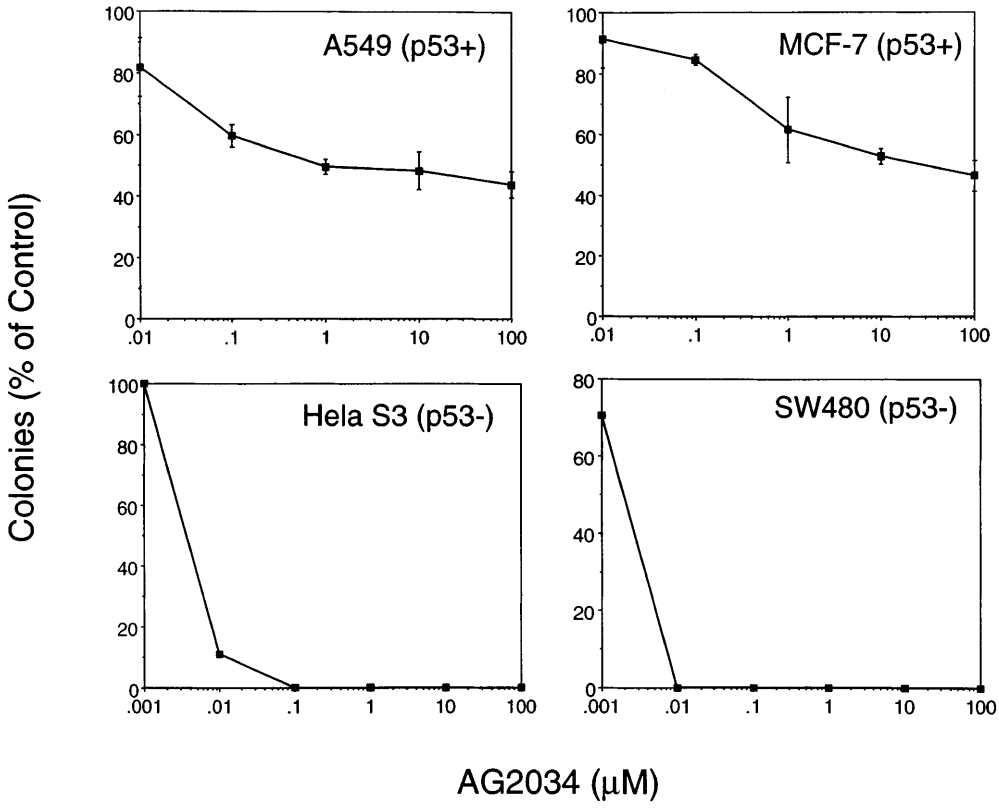

Fig. 3 Cytotoxicity of AG2034 as measured by clonogenic assays in A549, MCF-7, HeLa/S3 and SW480 cells. The medium contained 200 n*M* folic acid for MCF-7 cells and 50 n*M* folic acid for the other cell lines. Colonies were stained with 1% methylene blue before counting

Table 1 The effect of AG2034 on cellular ribonucleoside 5′-triphosphates. Cells were treated with 10 μ*M* AG2034 at 37 °C for 24 h, and nucleotides were extracted and measured as described in Methods

Cell line	Ribonucleotide pools in treated cells as percent of control			
	UTP	CTP	ATP	GTP
HeLa/S3	71	64	26	57
A549	91	64	40	76
MCF-7	56	57	41	52

Many studies have shown that p53 mediates a G1 arrest in cells following treatment with DNA-damaging agents, and it is generally believed that DNA damage is the upstream signal that triggers p53-mediated cell cycle arrest. When the p53-competent A549 and H460 cell lines were treated with 10 μ*M* AG2034 for 48 h at 37 °C, an increased proportion of cells in the G1 phase was observed. However, when cell lines lacking a functional G1 checkpoint (SW480, HeLa/S3) were similarly treated, the fraction of cells in G1 phase declined, and a large accumulation of cells was seen in S phase (Fig. 4). A subsequent experiment in which HeLa/S3 cells were treated with 10 n*M* AG2034 followed cell cycle distribution for 168 h. After 48 h, the fraction of cells in G1 declined, and cell accumulation was seen in S phase. By 1 week after the start of treatment, most of the accumulated S-phase cells then died, and most of the remaining cells were in the G2 + M compartment (Fig. 5).

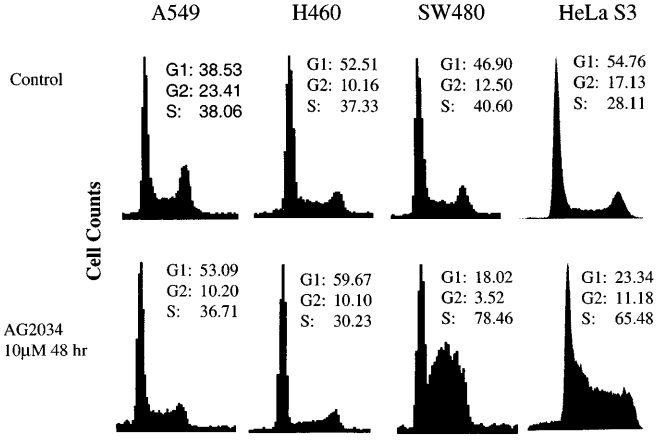

Fig. 4 Cell cycle effects of AG2034. All cell lines were treated for 48 h with AG2034 at 10 μ*M* in MEM containing 50 n*M* folic acid. Cells were stained with propidium iodide and analyzed by flow cytometry as described in Methods

Other classes of antifolates that induce a G1 cell cycle block have been shown to cause DNA strand-breaks, so it is believed that the p53-dependent checkpoint is triggered by these agents. We used the alkaline elution assay to examine for the presence of DNA damage after a 24-h treatment with AG2034 (Fig. 6). Figure 6 (left) shows the effect of the positive control compound, AG337, an inhibitor of thymidylate synthase. After a 24-h treatment of L1210 cells with AG337, a dose-dependent increase in the elution rate of DNA was seen, indicating

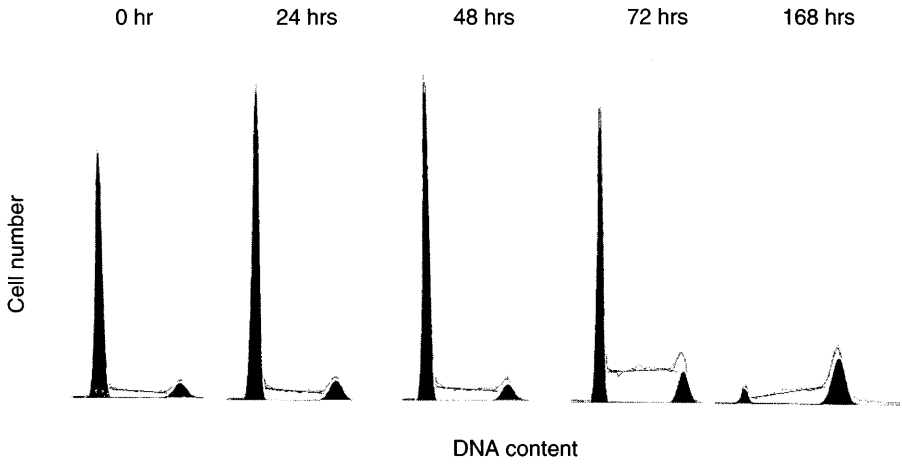

Fig. 5 Flow cytometric analysis of HeLa/S3 cells undergoing continuous treatment with 10 n*M* AG2034. The culture medium contained 50 n*M* folic acid

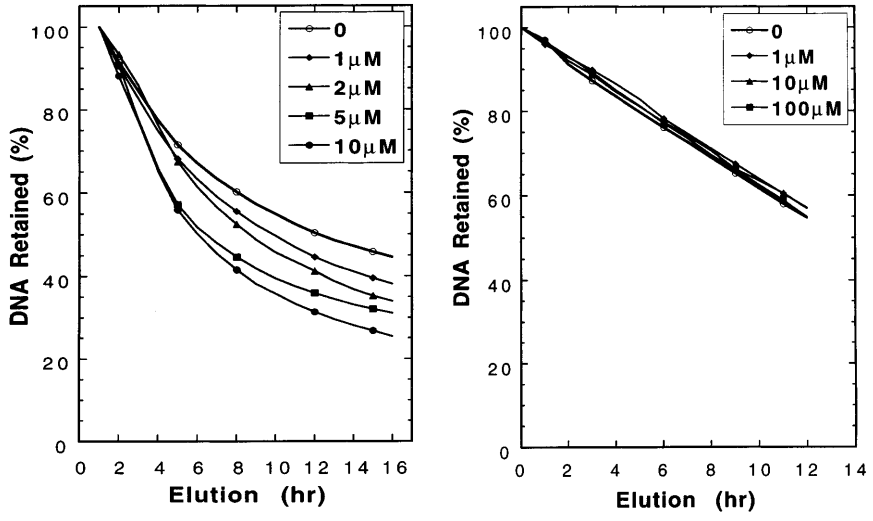

Fig. 6 Alkaline elution of DNA from L1210 cells treated for 24 h with the thymidylate synthase inhibitor AG337 (*left*) and with AG2034 (*right*)

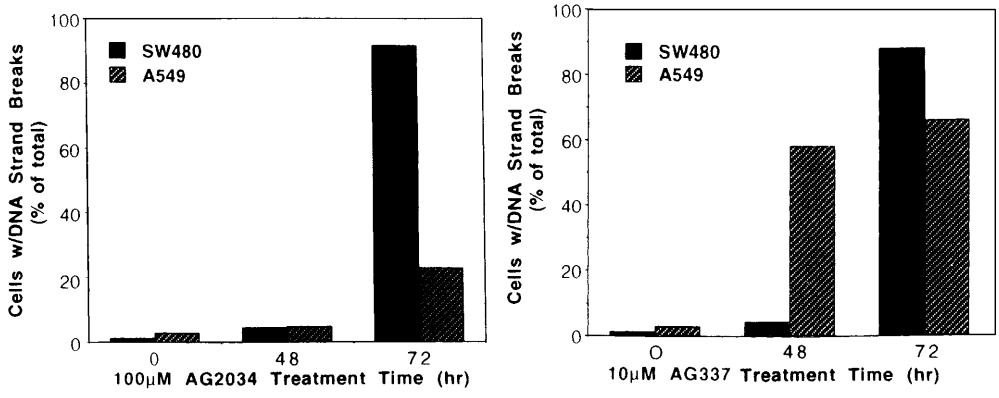

Fig. 7 Measurement of DNA strand breaks by the TUNEL assay in A549 and SW480 cells treated with AG2034 (*left*) and AG337 (*right*). Experimental details are provided in Methods

the presence of strand-breaks. However, after treatment with AG2034 for 24 h, at concentrations up to 100 μ*M* (Fig. 6, right), no breaks were seen. This study utilized an equimolar *RS* mixture of diastereoisomers differing in the configuration at the 6-position (see Fig. 1). All other studies described in this report used the pure 6*S* diastereoisomer. Another approach to measuring DNA strand-breaks is the TUNEL assay [9], in which DNA nicks are labelled with deoxyuridine which is then visualized with a fluorescent antibody. Figure 7 (right) shows the effect of 10 μ*M* AG337 in this system. As with other thymidylate synthase inhibitors, AG337 gave early DNA strand-breaks in the A549 cell line, which has a functional G1 checkpoint, whereas in the SW480 cell

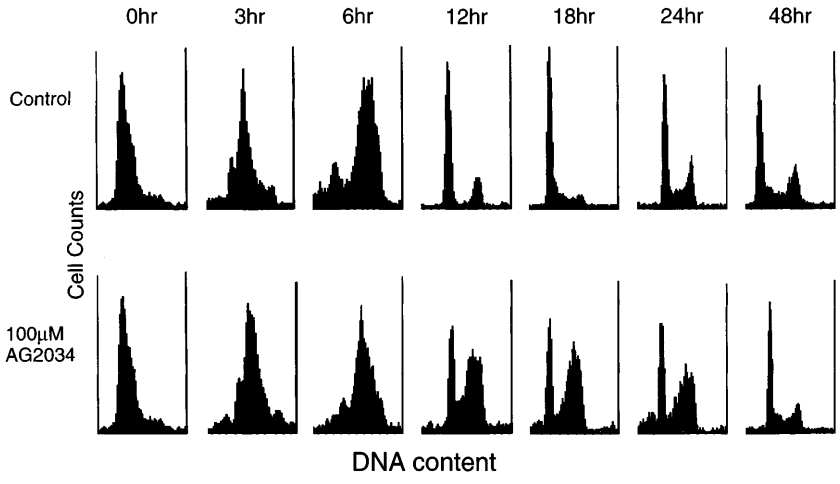

Fig. 8 The effect of AG2034 (100 μM) on the cell cycle of synchronized cultures of HeLa/S3 cells following release from a thymidine blockade. The synchronization procedure is described in Methods. After the removal of the thymidine, samples were analyzed at various times by propidium iodide staining and flow cytometry

line, lacking a G1 checkpoint, DNA breaks were also seen, but not until 72 h after the start of treatment. AG2034 at 100 μM gave no significant DNA damage at 48 h in either cell line (Fig. 7, left). By 72 h the SW480 line had suffered DNA damage in more than 90% of the cells, while the A549 line showed only about 20% of cells with DNA breaks.

In an attempt to clarify the course of cell cycle progression after AG2034 treatment, the effects of this inhibitor were studied in a culture of HeLa/S3 cells that had been synchronized at the G1/S boundary by thymidine blockade [9]. The results are shown in Fig. 8. When the thymidine was removed from a control culture, a large cohort of cells moved synchronously through S phase, and by 18 h was almost entirely back in G1, after which it progressively lost synchrony (Fig. 8, top). A similarly synchronized culture that was maintained in the continuous presence of 100 μM AG2034 moved much more slowly through S phase, with some cells in G2 and many cells still in mid to late S phase after 24 h (Fig. 8, bottom).

Discussion

Our interpretation of these results is that AG2034 caused purine depletion in all the cell lines, but that the cells responded differently to purine depletion depending upon the presence or absence of a G1 checkpoint. In cells possessing a functional G1 checkpoint, inhibition of the purine *de novo* pathway caused G1 arrest, but the arrested cells remained viable. Cells that were already in S phase died, so that treatment with AG2034 caused about 50% cell kill. In contrast, in cells that did not have a G1 checkpoint, most of the cells progressed into S phase, or into G2, where they died. AG2034 thus caused a predominantly cytostatic effect in cells with a functioning G1 checkpoint, and a highly cytotoxic effect in cells lacking this checkpoint. Since AG2034 is a highly selective GARFT inhibitor that causes no other known metabolic perturbations, these cell cycle effects must be the result of purine depletion. These results are in sharp contrast to the reported effects of other antimetabolites, e.g. inhibitors of thymidylate synthase or dihydrofolate reductase, where cell cycle arrest leads to apoptotic cell death [3, 7] and where $p53^-$ cells are generally less drug-sensitive [10].

It has been recently proposed that, in addition to DNA strand-breaks, the G1 checkpoint could be triggered by ribonucleotide depletion [6, 16]. Linke et al. have shown that normal human skin fibroblasts treated with mycophenolic acid, an inhibitor of IMP dehydrogenase that selectively depletes guanine nucleotides, arrest in G1 but remain viable and morphologically normal [6]. However, cells that have been transfected with human papillomavirus type 16 E6 protein, which inactivates p53, arrest in S phase. However, alanosine (an inhibitor of adenylosuccinate synthetase), which selectively depletes adenine nucleotides, gives S phase accumulation in fibroblasts regardless of their p53 status. Methylmercaptopurine riboside (MMPR), an inhibitor of PRPP amidotransferase, gives a G1 block regardless of the p53 status of the cells [6]. However, MMPR can be converted within cells to 6-mercaptopurine and 6-thioguanine, which have complex effects when incorporated into RNA and DNA.

A recent study by Tonkinson et al. [13] has found that human leukemia cells treated with a thymidylate synthase inhibitor are arrested at the G1/S boundary, while a GARFT inhibitor causes an increased number of cells in S phase. This is in agreement with our conclusions from the present study, though the high levels of folic acid used by Tonkinson et al. would have limited the amount of cytotoxicity observed. The effect of AG2034 seen in our present study resembles that of mycophenolic acid or PALA in that it gave a G1 blockade in cells with a functional G1 checkpoint, in the absence of DNA damage, while allowing cells without a G1 checkpoint to progress into S phase. These results thus support the "metabolite sensor" function of p53 proposed by Wahl and his colleagues (Linke et al. [6], Yin et al. [16]). However, AG2034 may differ from these other agents in

that low concentrations give almost total cell death among those $p53^-$ cells that progress into S phase. While numerous other antipurine drugs have been developed as anticancer agents, most of these compounds have been analogues of purine nucleosides or nucleobases. As such, they are incorporated into nucleic acids, and (like MMPR) cause multiple biochemical effects in addition to inhibition of purine biosynthesis. To selectively destroy $p53^-$ cells, a drug must possess two attributes: it must cause a metabolic perturbation that triggers the G1 checkpoint, and it must be lethal to cells in the S or G2 phases. Selective inhibitors of GARFT, such as AG2034, appear to possess the required properties, and should therefore be cytostatic to normal cells and selectively cytotoxic to $p53^-$ tumor cells. AG2034 is currently undergoing clinical trials as a potential anticancer drug.

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