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ORIGINAL ARTICLE

The depletion of cellular ATP by AG2034 mediates cell death or cytostasis in a hypoxanthine-dependent manner in human prostate cancer cells

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Abstract
Purpose 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-amino-l-glutamic acid (AG2034), is a classical antifolate, an analog of folic acid that has been shown to be an excellent inhibitor of glycinamide ribonucleotide formyltransferase (GARFT), ultimately inhibiting the de novo synthesis of purines. We examined the eVect of this drug on cell proliferation, steady-state ATP levels, de novo and hypoxanthine salvage ATP synthesis, and on the phosphorylation of AMP kinase, in two diVerent androgen independent prostate cancer cell lines, DU145 and PC-3.
Methods Cells were maintained in culture medium containing 10 nM 5-methyl tetrahydrofolate supplemented with or without 1.7 μM hypoxanthine and 1.5 μM thymidine. Cytotoxicity of AG2034 was determined by clonogenic assays. AG2034-induced inhibition of cell proliferation was determined by electronic counting of cells over varying periods of time. Total cellular AMP and ATP pre- and post-drug treatment was quantified by reverse-phase HPLC. [^{14}C]-Glycine incorporation and [^{3}H]-hypoxanthine conversion into ATP were determined by liquid scintillation counting of HPLC isolated ATP fractions. The phosphorylation of AMP kinase (AMPK) was detected by western blotting.
Results In the absence of 1.7 μM hypoxanthine, AG2034 was cytotoxic to both DU145 and PC-3 cells. In its presence, the cells remained cytostatic for 14 days after which time DU145 but not PC-3 re-initiated growth that was maintained for 35 days even though steady-state levels of ATP in both cell lines remained depleted and [^{14}C]-glycine incorporation into ATP was inhibited by >95%. Salvage purine synthesis as measured by incorporation of [^{3}H]-hypoxanthine into ATP was maintained in both cell lines albeit to different levels. When AG2034 was added to the culture medium in the presence or absence of 1.7 μM hypoxanthine, cellular ATP levels were reduced by 80% within 24 h in both the cell lines. In the absence of hypoxanthine, the AMP/ATP ratio in PC-3 cells increased by 38% and was accompanied by a modest increase in the level of phosphorylated AMPK; no increase was observed in the presence of hypoxanthine where the AMP/ATP ratio increased by approximately 10%. Under these same culture conditions, the AMP/ATP ratio in DU145 cells in the absence of hypoxanthine increased by 60% and was accompanied by a large increase in phosphorylated AMPK. In the presence of hypoxanthine however, even though the AMP/ATP ratio increased 2.5-fold, phosphorylated AMPK levels did not increase.
Conclusions The cytostatic versus the cytotoxic effect of AG2034 on PC-3 and DU145 cells is mediated by the presence or absence, respectively, of physiological levels of hypoxanthine (1.7 μM) in the media. The ability of DU145 as opposed to PC-3 cells to proliferate in the presence of AG2034 is independent of the intracellular concentration of ATP. Activation of the AMPK signaling pathway in drug-treated PC-3 and DU145 cells is cell line dependent and independent of the AMP/ATP ratio.

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Abbreviations

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-thienoylamino-L-glutamic acid
FBS	Fetal bovine serum
csd-FBS	Charcoal stripped and dialyzed-FBS
GARFT	Glycinamide ribonucleotide formyltransferase
FPGS	Folylpolyglutamate synthase
DPBS	Dulbecco's phosphate buffered saline solution
HPX	Hypoxanthine
FA-Plus medium	MEM/F12 medium (deficient in hypoxanthine, thymidine, folate, L-glutamine and phenol red) supplemented with 10% csd-FBS, 1.7 μM hypoxanthine, 1.5 μM thymidine, 0.05 nM R1881 hormone, 10 nM 5-methyl tetrahydrofolate, penicillin (100 units/ml)/streptomycin (100 μg/ml) and 2 mM L-glutamine
FA medium	FA-Plus without hypoxanthine and thymidine

Introduction

It is generally thought that when compared to "normal cells", cancer cells typically display an increase in anabolic processes. They frequently overexpress enzymes driving lipid synthesis and many involved in the glycolytic pathway [11, 21]. Since all these processes are energy requiring, interfering with mechanisms controlling cellular energy balance has been considered a promising strategy for the development of novel chemotherapeutic drugs [11, 21]. Since two of the enzymes required for the de novo synthesis of purine and ultimately ATP, glycinamide ribonucleotide formyltransferase (GARFT) and 5-aminoimidazole-4-carboximide ribonucleotide transformylase (AICART), require the folate cofactor N^{10}-formyl tetrahydrofolate, a focus of this approach has been the design of drugs that inhibit de novo purine synthesis utilizing folic acid antagonists, i.e., antifolates. The use of antifolates as anti-cancer drugs is supported by a substantial body of laboratory studies and clinical experience [7, 15, 24]. As folate metabolism is complex and many well-known antifolates have multiple targets [1, 2, 10, 20], efforts have been made to develop more specific agents. An example is AG2034, an analog of folic acid that was designed based on the crystal structure of *E. coli* GARFT, and the GARFT domain of the human tri-functional enzyme [6] for which it is a very effective and specific inhibitor [6]. AG2034 is also an excellent substrate for polyglutamylation by folylpolyglutamate synthase (FPGS) and is transported inwards primarily by the reduced folate carrier, ultimately inhibiting de novo purine synthesis [5].

The intrinsic challenge of inhibiting de novo purine synthesis as an effective chemotherapeutic strategy is significant, given the ability of mammalian cells to utilize salvage mechanisms to generate purine rings for AMP and GMP synthesis from substrates such as inosine or hypoxanthine in the media. Nevertheless, the notion that cancer cells rely on de novo as opposed to salvage synthesis of purines [17] suggests that a therapeutic window might be gained if de novo synthesis was inhibited resulting in tumor as opposed to normal cell kill as a consequence of enhanced ATP depletion in the tumor. One of the major questions underlying this concept, however, focuses on the capacity of normal and tumor cells to switch from de novo to salvage synthesis and additionally on the metabolic consequences to the cell from such a switch, if any.

Although previous studies have documented the effectiveness of AG2034 against a variety of tumor cell lines and xenografts [3, 6, 8, 9, 30], little information is available concerning the ability of this drug to inhibit growth or to induce cell kill in prostate cancer cell lines. Prostate cancer cells are unique in that they display metabolic characteristics similar to normal cells. That is they rely primarily upon Krebs cycle as opposed to aerobic glycolysis for the production of ATP [14], whereas most tumor types utilize aerobic glycolysis for the synthesis of ATP and display a low respiration rate [27]. In addition it has been reported that one of the most consistent and significantly dysregulated cohort of genes associated with prostate cancer are those involved with purine biosynthetic pathways [23].

To better understand how the AG2034 enforced inhibition of de novo purine synthesis affects prostate cancer cell lines in culture, we have examined its effect on steady-state levels of AMP and ATP, de novo ATP synthesis via [^{14}C]-glycine incorporation, salvage synthesis of ATP via the conversion of [^{3}H]-hypoxanthine, cell proliferation and on the activation of the AMPK signaling pathway. These studies were carried under culture conditions where the levels of reduced folate and hypoxanthine were maintained in the physiological range, 10 nM 5-methyl tetrahydrofolate and 1.7 μM, respectfully, to avoid the known ability of these compounds at high concentrations to confer resistance to AG2034 [30]. We utilized two prostate cancer cell lines, DU145 and PC-3, because they have been extensively characterized, possess different genotypes and growth characteristics, and both are androgen independent. PC-3 is p53-null but expresses the functional Rb protein [31] whereas DU145 cells express

mutant p53 and are Rb protein negative [4, 31]. Our results show that these two tumor cell lines utilize both de novo and salvage pathways for ATP synthesis, and that the presence of hypoxanthine at physiological levels in the media promotes cytostasis as opposed to cytotoxicity in response to AG2034 treatment. Additionally, the effect of AG2034 at inhibiting de novo purine synthesis occurs rapidly, independent of the presence or absence of 1.7 μM hypoxanthine, resulting in a prolonged depletion of ATP in both PC-3 and DU145 cells, alike. We also present evidence showing that although both cell lines are androgen independent, DU145 cells but not PC-3 can proliferate in the absence of de novo purine synthesis, enforced by the presence of AG2034, even though the steady-state level of ATP in both cell lines remains depleted to essentially the same degree. Finally during drug treatment in the absence of hypoxanthine, depletion of ATP is associated with a substantial phosphorylation of AMPK in DU145 but not in PC-3 cells even though the AMP/ATP ratio increases to the same point in both. In the presence of hypoxanthine however, increased AMPK phosphorylation was not observed in either cell line although the AMP/ATP ratio in DU145 cells increased by 2.5-fold while that in PC-3 cells did not change significantly.

Materials and methods

Chemicals

DMEM/F12 was obtained from Biofluids/Biosource (Rockville, MD, USA) and fetal bovine serum (FBS) from Hyclone (Logan, UT, USA). Hypoxanthine-, thymidine-, folate-, L-glutamine- and phenol red-free MEM/F12 medium, trypsin/EDTA, Dulbecco's Phosphate buffered saline solution (DPBS), and penicillin G sodium (10,000 units/ml)/streptomycin sulfate (10,000 μg/ml) were from Invitrogen/Gibco whereas charcoal-stripped and dialyzed-FBS (csd-FBS) was obtained from Valley Biomedical (Winchester, VA, USA). Methyltrienolone, also known as R1881 hormone was purchased from Perkin Elmer. [^{14}C(U)]-$H_2N\mathbf{C}H_2\mathbf{C}OOH$ ([^{14}C]-glycine, 95 mCi/mmol) and [2,8-^{3}H]-hypoxanthine ([^{3}H]hypoxanthine, 27.8 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA, USA). The BCA protein assay kit was obtained from Cole-Parmer Instrument Company (Vernon Hills, IL, USA). AG2034 was a kind gift from Pfizer (Groton, CT, USA). All other chemicals and reagents were obtained from Sigma-Aldrich (USA), unless otherwise indicated.

Cell culture

The human prostate cancer cell lines (DU145 and PC-3) were purchased from American Type Culture Collection (Bethesda, MD, USA), and were initially cultured for 3 days in DMEM/F12 medium supplemented with 10% FBS and penicillin (100 units/ml)/streptomycin (100 μg/ml). These cells were then maintained in FA-Plus or FA medium for a minimum of 3 weeks before all experimental procedures. FA-Plus contains MEM/F12 medium (deficient in hypoxanthine, thymidine, folate, L-glutamine and phenol red) supplemented with 10% csd-FBS, 1.7 μM hypoxanthine, 1.5 μM thymidine, 0.05 nM R1881 hormone, 10 nM [6S]5-methyl tetrahydrofolate, penicillin (100 units/ml)/streptomycin (100 μg/ml) and 2 mM L-glutamine. FA medium is FA-Plus without hypoxanthine and thymidine. Cell viability measurements were determined by clonogenic assays. All cells were cultured as monolayers in a humidified environment of 5% CO_2/95% air at 37°C.

Dose response assay

The ED50, defined as the dose required to reduce cell growth to 50% of controls after 120 h drug treatment, was used as a measure of drug sensitivity. Cells (1×10^4) were plated in 24-well plates and allowed overnight to adhere. Thereafter, AG2034 was added continuously in either FA or FA-Plus medium for 120 h and renewed every 2–3 days. Cells were then washed with DPBS, harvested by trypsinization and an aliquot of the homogenous cell suspension was counted electronically using a Beckman Coulter Counter.

Cell counting assay

Based on results obtained from clonogenic assays, cells in log growth phase were exposed to 50 nM AG2034. Cells ($\sim 1.0 \times 10^4$ per well) were plated in 6-well plates and allowed a period of 12–24 h for cells to adhere to plates. Thereafter, cells were incubated with/without 50 nM AG2034 in FA or FA-Plus medium for varying periods of time. Culture medium was renewed with freshly prepared medium with or without 50 nM AG2034 every 2–3 days. Cells were harvested by trypsinization and an aliquot of the homogenous cell suspension was counted electronically using a Beckman Coulter Counter.

Clonogenic survival assay

Cells (1×10^3) were plated in 6-well plates in FA or FA-Plus media. After 24 h, cells were exposed to varying concentrations of AG2034 for a further 24 h. Thereafter, the culture media was completely renewed with drug-free FA or FA-Plus media with media renewal every 2–3 days for an additional 14 days. Cell monolayers were then washed with DPBS, fixed in 1% crystal violet in absolute methanol. Cells were washed once with DPBS, air-dried and counted (colonies > 30 cells). Clonogenic survival results were

corrected for differences in plating efficiency: [(number of colonies/number of colonies of untreated cells) × 100]; for the various culture conditions.

Cell treatments prior to ATP and [^{14}C]-glycine incorporation assay

In these experiments, cells (2×10^6) were plated in 175 cm^2 culture flasks in FA-Plus or FA medium and allowed 18–24 h to adhere to the surface. Fresh medium supplemented with 0–1 μM AG2034 was then added and cultures incubated for another 24 h. Later, cell monolayers were washed with DPBS and this was followed by the addition of drug-free fresh medium containing 0.064 μCi/ml of [^{14}C]-glycine. Cultures were then incubated for a further 24 h prior to the determination of ATP levels and [^{14}C]-glycine incorporation into ATP. In some experiments, cells were treated with 50 nM AG2034 for varying times. Thereafter, cell monolayers were washed twice with DPBS, the culture medium replaced with AG2034-free medium containing 0.064 μCi/ml of [^{14}C]-glycine and then incubated for a further 24 h. In other experiments, the immediate effect of AG2034 was assessed by incubating cells in fresh medium containing 0.064 μCi/ml of [^{14}C]-glycine with/without 50 nM AG2034 for varying periods of time over 24 h. For the last set of experiments, cells were incubated with 50 nM AG2034 continuously over 35 days and media was supplemented with 0.064 μCi/ml of [^{14}C]-glycine for the final 24 h. For this set of experiments, culture media was renewed every 2–3 days.

Assay to determine the conversion of [^{3}H]-hypoxanthine into ATP

Cells (2×10^6) were plated in 175 cm² culture flasks in FA-Plus medium and allowed to adhere to the surface overnight. Afterwards, cells were incubated with 50 nM AG2034 continuously over 21 days and media supplemented with 0.8 μCi/ml of [^{3}H]-hypoxanthine for the final 24 h. Culture media was renewed every 2–3 days. This was followed by nucleotide analysis.

Nucleotide extraction and analysis

After the appropriate incubation times, cell monolayers were washed twice with ice-cold DPBS. Thereafter, cells were harvested by trypsinization followed by centrifugation at 3,000*g* at ≤4°C for 10 min. Cell pellets were then washed in ice-cold MEM/F12 and centrifuged at 10,000*g* at ≤4°C for 10 min. Cells were lyzed by the addition of trichloroacetic acid (0.73 M, final concentration), vortexed and then incubated on ice for 2 min. The resulting suspension was cleared by centrifugation at 15,000*g* for 2 min. An aliquot of the supernatant was neutralized with ice-cold KOH, mixed followed by a brief spin. Aliquots of the resulting supernatant were stored at −80°C for 1–5.5 weeks prior to nucleotide analysis. Total cellular AMP and ATP were quantified by reverse-phase HPLC of trichloroacetic acid extracts using a Vydac C18, reverse-phase column, as previously described [25]. Protein concentration of untreated cells (controls) was determined spectrophotometrically using the BCA protein assay kit.

[^{14}C]-Glycine incorporation and [^{3}H]-hypoxanthine conversion assays

[^{14}C]-glycine incorporation and [^{3}H]-hypoxanthine conversion into ATP were determined by collecting ATP fractions after nucleotide separation by HPLC. An aliquot of the ATP fraction was assayed for radioactivity by liquid scintillation counting. [^{14}C]-glycine incorporation into ATP is expressed as a percentage of the total incorporated radioactivity into the ATP fractions of the respective untreated cells (controls). [^{3}H]-hypoxanthine conversion into ATP is expressed as pmol/mg protein.

Western blot analysis

Cells were treated for varying periods of time with 50 nM AG2034 and then lysed in ice-cold RIPA buffer (Phosphate buffered saline, 3.5 mM sodium dodecyl sulfate, 11.6 mM sodium deoxycholate, 16.3 mM Nonidet P-40) supplemented with 1 mM Na_3VO_4, 0.57 mM phenylmethylsulfonyl fluoride, and 7.7 μM aprotinin. Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL, USA) using bovine serum albumin as the standard. Equal amounts of protein were mixed with SDS loading buffer, boiled and separated on 10% Tris–HCl gels. Proteins were then transferred onto PVDF membranes (Biorad, Hercules, CA, USA), blocked with 5% nonfat dry milk for total proteins and 5% bovine serum albumin for phosphorylated proteins. Membranes were probed with specific antibodies against AMPKα, phospho-AMPKα (Thr 172) (Cell signaling technology, Beverly, MA, USA) and β-actin (Oncogene, Boston, MA, USA). The appropriate horseradish peroxidase-conjugated secondary antibodies were used and developed using the HyGLO chemiluminescent detection reagent (Denville Scientific, Metuchen, NJ, USA).

Data analysis

Data shown are from three independent experiments. Comparisons were made by one-way analysis of variance (ANOVA) using MINITAB statistical software, version 13.32. Post hoc comparisons of means were made using Tukey's pairwise method. Differences were considered significant at $P < 0.05$.

Results

Inhibition of cell proliferation in AG2034-treated prostate cancer cells

Growth inhibition assays carried out over a 120 h period with continuous exposure, allowed us to estimate an ED50 of approximately 4 nM for AG2034 in both DU145 (Fig. 1a) and PC-3 (Fig. 1b) human prostate cancer cell lines in both FA and FA-Plus media (Fig. 1). However, even at drug concentrations in excess of 40 nM, the number of cells remaining on the plates at 120 h was well in excess of the number plated suggesting that the drug was acting as a cytostatic as opposed to a cytotoxic agent. Therefore, we sought to determine what effects would be observed with exposure times in excess of 120 h. Cells (1.0×10^4) were treated with 50 nM AG2034 in either FA-Plus or FA culture media and cell counts were determined electronically at various points in time over 35 days. As shown in Fig. 2, treatment with AG2034 caused the loss of DU145 (Fig. 2a) and PC-3 cells (Fig. 2b) after 7 days of exposure but only in FA medium. The reduction in cell numbers is attributed to the lost ability of these cells to attach to plates, a phenomenon characteristic of cell death for normally adherent cells. There was no significant difference in cell numbers between

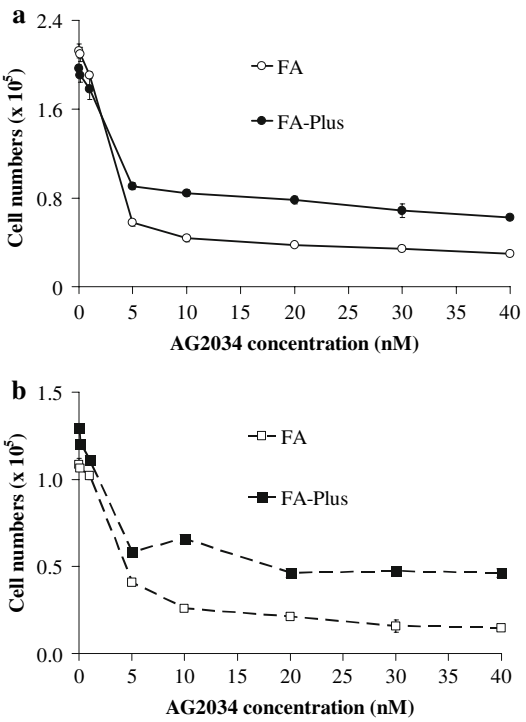

Fig. 1 Dose-response curves to AG2034 under conditions of continuous exposure for 120 h for ED50 determinations. Cells **a** DU145 and **b** PC-3 were plated at 1×10^4 cells/well overnight before the drug was added for 120 h in fresh medium either FA or FA-Plus. Thereafter, cell numbers were counted electronically

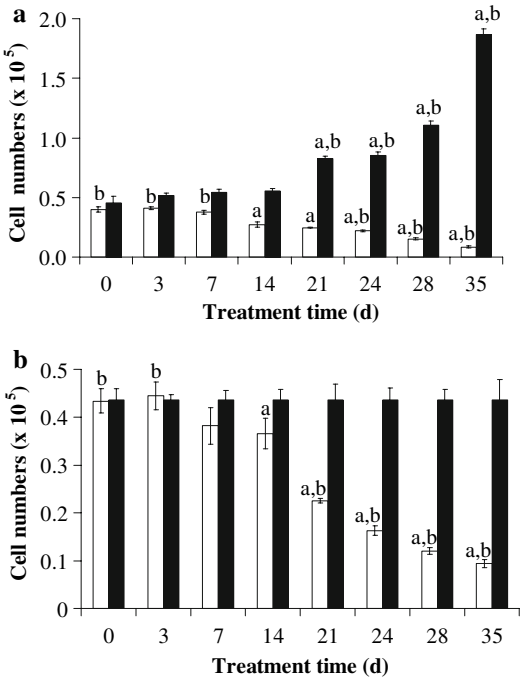

Fig. 2 AG2034 induces growth inhibition in human prostate cancer cells. Androgen independent cells **a** DU145 and **b** PC-3 were plated at 1×10^4 cells/well overnight and then fresh medium either FA (*open columns*) or FA-Plus (*closed columns*) containing 50 nM AG2034 with (FA-Plus) or without (FA) 1.7 μM hypoxanthine/1.5 μM thymidine was added. Cell density was determined electronically at the indicated times shown with culture medium renewal every 2–3 days. Values represent mean ± SD of three independent counts. **a** $P < 0.01$ versus day zero; **b** $P < 0.01$ versus day 14 by one-way ANOVA and post hoc comparisons by Tukey's test

DU145 and PC-3 cells with drug treatments for ≤7 days compared to untreated cells in FA medium. However, the cell number was significantly reduced with 14 days of drug treatment in FA medium and this steadily and rapidly decreased with time for 21 more days with a 79 and 78% reduction in cell numbers counted on day 35 compared to untreated cells (day 0) in DU145 and PC-3 cells, respectively. In FA-Plus media, DU145 and PC-3 cells remained cytostatic with AG2034 treatment for ≤14 and 35 days, respectively. Unexpectedly, DU145 cells began to proliferate after 14 days of continuous drug treatment and cell density proceeded to increase approximately linearly with respect to time by 82, 145 and 313% compared to untreated cells on days 21, 28 and 35, respectively (Fig. 2). Hence, in the presence of physiological concentrations of hypoxanthine, AG2034 acts as a cytostatic agent.

That AG2034 can be cytotoxic to both PC-3 and DU145 cells after 24 h of exposure, however, is demonstrated in Fig. 3. In the absence of hypoxanthine, PC-3 and DU145 cells exhibit LD50's of ~5 and 50 nM, respectively and no clones survived at concentrations in excess of 10 μM. In the presence of 1.7 μM hypoxanthine, the LD50's increased

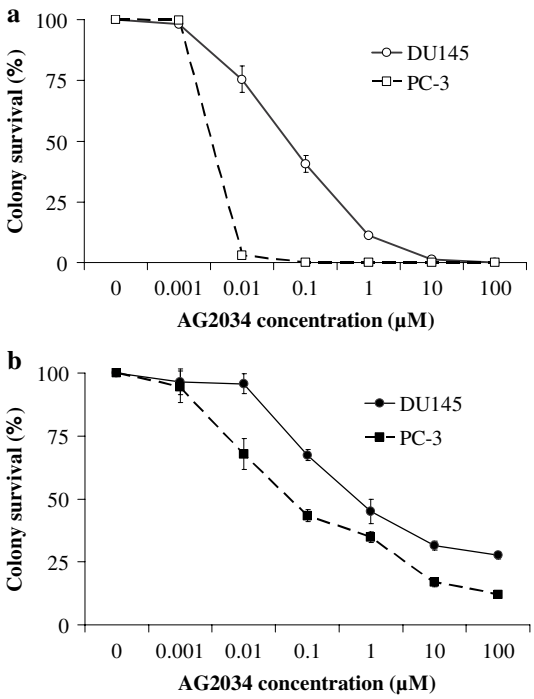

Fig. 3 AG2034 is cytotoxic to prostate cancer cells. Androgen independent cells with mutant p53 (DU145) and cells that are p53-null (PC-3) were cultured in FA (**a**) or FA-Plus (**b**) medium. Cells were seeded and allowed 24 h to adhere to plates. These cells were then exposed to varying concentrations of AG2034 for 24 h followed by drug withdrawal and the specific culture medium was renewed every 2–3 days for a further 14 days. Thereafter, cells were stained and counted as described in "Materials and methods". Data represent mean counts ± SD of three independent determinations

significantly to approximately 100 nM for PC-3 and about 1 μM for DU145. Moreover, significant numbers of colonies of both cell lines persisted at drug concentrations of 100 μM. In the case of DU145 it is likely that these persistent colonies are composed of cells that are able to proliferate after14 days of continuous exposure to drug as shown in Fig. 2a. While colonies of PC-3 cells also persisted in FA-Plus medium there are fewer of them and the cells present cannot reinitiate growth (Fig. 2b).

ATP depletion studies

In order to assess the effect of AG2034 on steady-state ATP levels, DU145 and PC-3 cells were first exposed to different drug concentrations for 24 h, and then incubated in drug-free medium for a further 24 h. As shown in Fig. 4a, d, 24 h treatment with 50 nM AG2034 reduced the steady-state level of ATP in both DU145 and PC-3 cells from approximately 55 to 10 nmol/mg protein. Increasing the drug concentration to 1 μM did not cause any additional reduction in ATP levels. Interestingly, similar results were obtained in both FA and FA-Plus media indicating that during the 48 h time period of these experiments, neither cell line was able to utilize purine salvage to maintain intracellular ATP levels. The time course of the reduction in ATP levels was determined by treating cells with 50 nM drug at intervals from 4 to 120 h. As indicated in Fig. 4b, e after 4 h of drug exposure, the ATP levels in DU145 and PC-3 cells cultured in FA media decreased significantly ($P < 0.01$) from

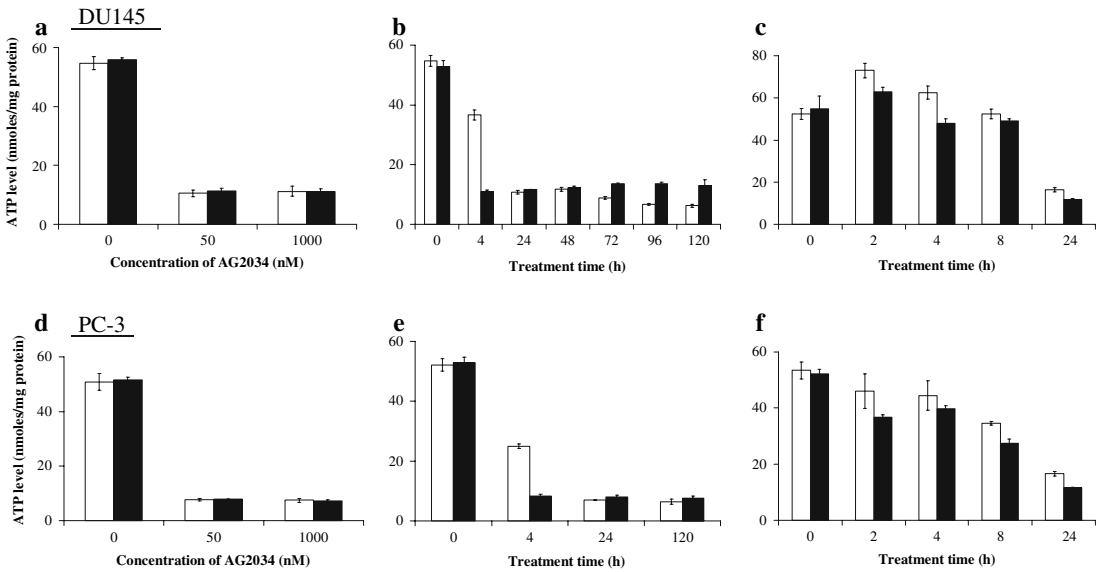

Fig. 4 The effect of AG2034 on ATP depletion in two prostate cancer cells. Cells (2×10^6) **a**–**c** *DU145* and **d**–**f** *PC-3* were exposed to AG2034 as described in "Materials and methods". ATP levels of trichloroacetic acid extracts post AG2034 treatment were quantified by reverse-phase HPLC. Cells were maintained in either FA (*open columns*) or FA-Plus (*closed columns*) medium. Data represent mean ± SD of three independent determinations. **a**, **d** Cells were pre-treated with 0–1,000 nM AG2034 for 24 h and then in drug-free medium for a further 24 h; **b**, **e** cells were pre-treated with 50 nM AG2034 for 0–120 h and then in drug-free medium for a further 24 h and **c**, **f** cells were exposed to 50 nM AG2034 for 0–24 h and ATP was analyzed immediately after the indicated time

approximately 55–37 and 25 nmol/mg protein, respectively. When grown in FA-Plus media these values decreased further to 10 nmol/mg protein for both cell lines. Hence the presence of hypoxanthine in the media actually enhanced the rate at which depletion of intracellular ATP occurred in both cell lines. After 24 h of drug exposure in either FA or FA-Plus medium, the steady-state level of ATP in PC-3 cells remained constant at ~8 nmol/mg protein. DU145 cells, however, behaved differently. While the ATP level remained constant through 48 h of drug treatment, in both FA and FA-Plus media, it began to decrease in FA media and by 120 h of drug exposure had decreased to approximately 6 nmol/mg protein. In FA-Plus media however, the level of ATP remained essentially constant at 10 nmol/mg protein from 48 h through 120 h of drug exposure. To more closely determine the short-term effects of drug treatment on ATP levels, cells were exposed to 50 nM drug for 2–24 h and ATP levels determined immediately. As shown in Fig. 4c, f after 2 h of drug treatment, the ATP levels in DU145 cells unexpectedly increased from 52 to 70 nmol/mg protein ($P < 0.01$), in FA media and from 53 to 62 nmol/mg protein ($P > 0.05$) in FA-Plus media, after which they decreased in a linear fashion to 16 and 12 nmol/mg protein ($P < 0.001$) in FA and FA-Plus, respectively, with 24 h of treatment. Under the same drug treatment conditions, no increase in ATP levels was observed in PC-3 cells regardless of the media used, and after 24 h of drug exposure the ATP levels had decreased to approximately the same levels reached in DU145 cells (Fig. 4c, f).

[^{14}C]-Glycine incorporation assay

To determine if the depletion of intracellular ATP levels in drug-treated cells was correlated with a commensurate decrease in de novo purine synthesis, the incorporation of [^{14}C]-glycine (a precursor in the de novo synthesis pathway) into ATP was measured. As indicated in Fig. 5a, d, treatment with 50 nM or 1 μM AG2034 for 24 h followed by growth in drug-free media for an additional 24 h, were equally effective in preventing the incorporation of [^{14}C]-glycine into ATP in both cell lines regardless of the media used. However, when the drug exposure time was reduced to 4 h, again followed by 24 h of growth in the absence of drug, the culture media did have an effect. As shown in Fig. 5b, e, the inhibition of [^{14}C]-glycine incorporation in both cell lines was significantly ($P < 0.01$) less in FA media than in FA-Plus, but this difference was not noted at longer treatment times. Hence, in the absence of 1.7 μM hypoxanthine, the ability of AG2034 to block de novo ATP synthesis during a 4 h exposure was suppressed. To show that this effect was not dependent upon the growth of the cells in drug-free media after exposure, PC-3 and DU145 cells were simultaneously exposed to 50 nM drug

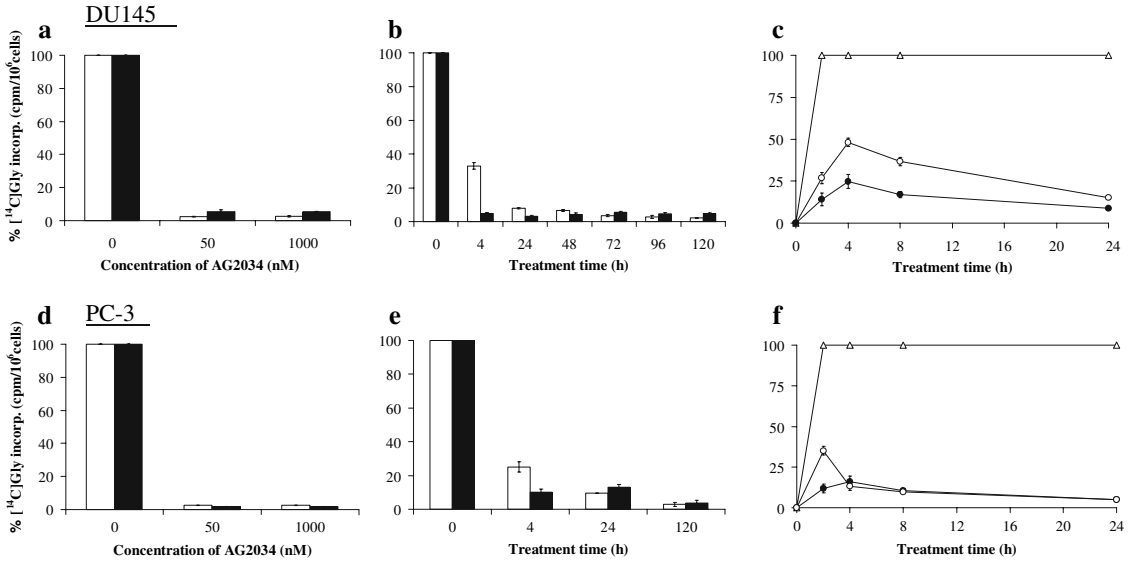

Fig. 5 The effect of AG2034 on [^{14}C]-glycine incorporation into ATP. Cells (2×10^6) **a**–**c** DU145 and **d**–**f** PC-3 were pre-treated with AG2034 as described in "Materials and methods". Post nucleotide separation of TCA extracts by HPLC, and fractions corresponding to ATP were collected and assayed for [^{14}C]-glycine radioactivity by liquid scintillation counting. *Open columns* denote cells maintained in FA while *closed columns* represent cells in FA-Plus medium. Data indicate mean% ^{14}C radioactivity in ATP (cpm per 10^6 cells) ± SD of three independent experiments. **a**, **d** Cells were pre-treated with 0–1,000 nM AG2034 for 24 h and then incubated in drug-free medium containing 0.064 μCi/ml of [^{14}C]-glycine for a further 24 h; **b**, **e** cells were pre-treated with 50 nM AG2034 for 0–120 h and then in drug-free medium containing [^{14}C]-glycine for a further 24 h and **c**, **f** cells were simultaneously exposed to 50 nM AG2034 and labeled with 0.064 μCi/ml of [^{14}C]-glycine for 0–24 h and ATP was analyzed immediately after the indicated time. *Open circles* denote the percentage of [^{14}C]-glycine incorporated into cells in FA medium and *closed circles* for those in FA-Plus medium, relative to that incorporated into AG2034-untreated cells (*open triangles*)

and [^{14}C]-glycine for various times from 2 to 24 h, harvested immediately, and [^{14}C] incorporation levels determined. As shown in Fig. 5c, f, after 2 h of drug exposure in FA media, [^{14}C]-glycine incorporation in both cell lines reached only 27–35% that of untreated controls. In FA-Plus medium the amount was approximately 14%. After 4 h of exposure in FA medium, [^{14}C]-glycine incorporation in DU145 cells continued to increase and reached 48% that for untreated controls and then linearly decreased with increasing treatment times. In FA-Plus media [^{14}C]-glycine incorporation reached about 24% of control values after 4 h of exposure and then it also decreased in a linear manner over 24 h of treatment to 15% of control values in FA media and to 9% in FA-Plus media. In PC-3 cells the drug effect was more pronounced and at 4 h of exposure regardless of the media used, [^{14}C]-glycine incorporation was approximately 13% that of controls decreasing to 5% over the course of the 24 h of treatment.

Metabolic studies with prolonged exposure of cells to AG2034

To further investigate the effect of drug treatment on these cells, total ATP levels were determined at various time points over 35 days of continuous exposure (Fig. 6). Without hypoxanthine in the culture medium (FA), cellular ATP concentrations remained depleted throughout the course of the experiment in both cell lines. The increase in ATP levels observed with cells cultured in FA-Plus medium suggests a striking adaptation of these cells (both PC-3 and DU145) to their environment and likely reflects their transition from reliance on de novo to salvage pathways in order to sustain ATP levels. Taking into account the difference observed in cell proliferation between cell lines with continuous drug exposure for up to 35 days (Fig. 2), our results suggest that the increase in ATP levels at >7 days, while able to support the maintenance of PC-3 is sufficient to allow the proliferation of DU145 cells. It is important to note that despite the marked increase in ATP levels with drug treatment for >7 days, [^{14}C]-glycine incorporation remained inhibited regardless of the presence or absence of hypoxanthine (Fig. 7), thus demonstrating the continual effectiveness of the drug throughout the course of the experiment. To determine the role that salvage might play in supporting the increase in steady-state ATP levels in the absence of de novo synthesis, we measured the incorporation of exogenously added [^{3}H]-hypoxanthine into ATP pre- and post- continuous drug exposure. As indicated in Table 2, in the absence of the drug, both cell lines are able

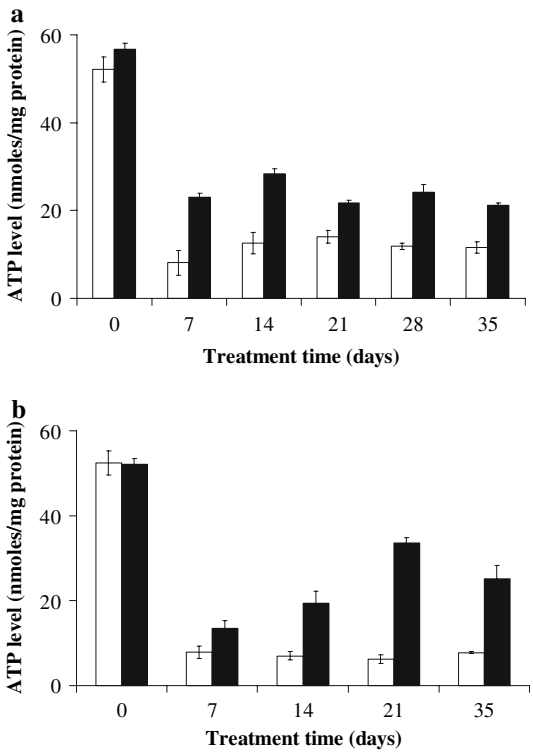

Fig. 6 The effect of AG2034 on ATP depletion in DU145 (**a**) and PC-3 (**b**) cells with continuous exposure to AG2034 for 35 days. *Open columns* denote cells maintained in FA while *closed columns* represent cells in FA-Plus medium. Values represent mean (%) ± SD of three independent determinations

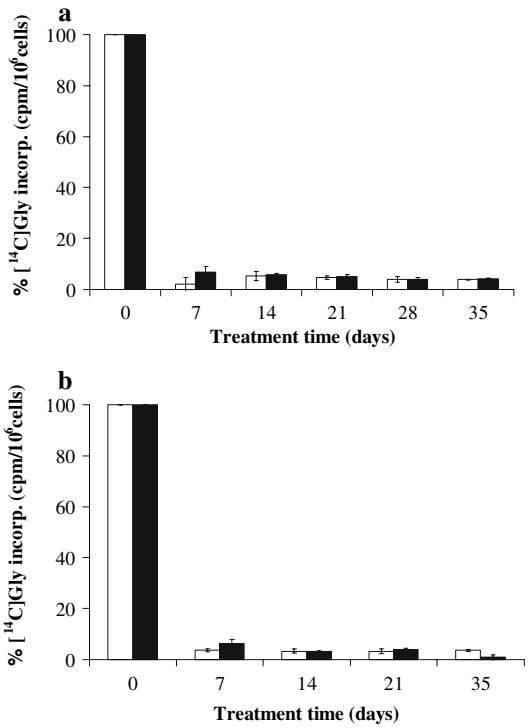

Fig. 7 The effect of AG2034 on [^{14}C]-glycine incorporation into cellular ATP with the continuous treatment of DU145 (**a**) and PC-3 (**b**) cells with AG2034 for 35 days. *Open columns* show cells maintained in FA while *closed columns* in FA-Plus medium. Values represent mean (%) ± SD of three independent determinations

to synthesis ATP via the salvage of hypoxanthine but DU145 cells are approximately 2.9-fold more effective in doing so. Unexpectedly, after 1 day of drug treatment, there was a marked ($P < 0.01$) reduction in the conversion of hypoxanthine into ATP by both PC-3 and DU145 cells possibly due to the inability of either cell line to compensate for the rapid loss of purines and therefore ATP production through the de novo synthetic pathway (Fig. 5). At 14 days of continuous exposure, however, both cell lines regained ($P < 0.01$) the ability to convert [^{3}H]-hypoxanthine to ATP producing 3.4 and 4.2 pmol of ATP/mg protein in DU145 and PC-3 cells, respectively. At 21 days, conversion of [^{3}H]-hypoxanthine into ATP decreased to 2.4 pmol of ATP/mg protein in DU145 cells while the amount in PC-3 continued to increase to 5.3 pmol/mg protein.

Activation of the AMPK pathway

Since an increase in the intracellular AMP/ATP ratio as a result of ATP depletion driven by heightened metabolic activity in mammalian cells is known to activate the AMPK signaling pathway, we sought to determine the extent to which the inhibition of de novo purine synthesis, which directly inhibits IMP synthesis in turn affecting both AMP and ATP levels might have on this pathway. As indicated in Table 1, after 24 h of drug treatment and in the absence of hypoxanthine, the AMP/ATP ratio was ~0.11 for both cell lines. As indicated in the Table this was a result of decreases in both AMP and ATP levels as expected. Interestingly, however, these changes were associated with a marked increase in the phosphorylation of AMPK in DU145 cells but not in PC-3 (Fig. 8). In the presence of hypoxanthine the AMP/ATP ratio increased dramatically in DU145 cells from 0.08 to 0.2, a 2.5-fold increase, while in PC-3 cells the increase was only 10% (Table 1). Yet in neither case was increased phosphorylation of AMPK observed (Fig. 8). Taken together, these results suggest that the response to changing AMP/ATP ratios caused by inhibition of de novo purine synthesis leads to profoundly different effects on the AMPK signaling pathway than those caused by metabolic stress and that the extent of these effects are cell line dependent.

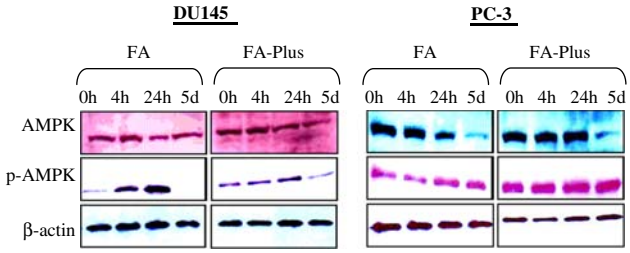

Fig. 8 *AMPK* activity is up-regulated in *DU145* and *PC-3* cells treated for 24 h with 50 nM AG2034 in FA media but not in the presence of hypoxanthine/thymidine (FA-Plus media)

Discussion

The first selective GARFT inhibitor tested in clinical trials was lometrexol but this agent demonstrated unacceptable levels of toxicity in humans [22]. Subsequently, AG2034 was rationally designed using knowledge of the X-ray crystal structure of GARFT from *Escherichia coli* and of the GARFT domain of the human tri-functional enzyme [6]. In preclinical studies, AG2034 was similar to lometrexol in terms of GARFT inhibition and substrate specificity for FPGS, but had a higher binding affinity for the membrane folate-binding protein [6]. Unfortunately, a phase 1 clinical

Table 1 The effect of AG2034 on AMP, ATP and AMP/ATP ratio in DU145 and PC-3 cells maintained in FA or FA-Plus media

Treatment time with AG2034 (h)	FA media			FA-Plus media		
	AMP (nanomol/mg protein)	ATP (nanomol/mg protein)	AMP/ATP	AMP (nanomol/mg protein)	ATP (nanomol/mg protein)	AMP/ATP
	DU145			DU145		
0	3.8 ± 0.81	54.8 ± 1.78	0.07 ± 0.017	4.2 ± 0.41	52.9 ± 2.02	0.08 ± 0.006
4	3.3 ± 0.14	36.7 ± 1.67*	0.09 ± 0.002	2.2 ± 0.31*	10.9 ± 0.68*	0.2 ± 0.032*
24	1.2 ± 0.05*	10.8 ± 0.58*	0.11 ± 0.002*	2.3 ± 0.38*	11.7 ± 0.02*	0.2 ± 0.033*
120	1.6 ± 0.19*	6.3 ± 0.53*	0.26 ± 0.012*	2.6 ± 0.24*	13.0 ± 1.94*	0.2 ± 0.013*
	PC-3			PC-3		
0	4.2 ± 0.30	52.1 ± 2.08	0.08 ± 0.003	4.2 ± 0.25	52.8 ± 1.97	0.08 ± 0.002
4	2.0 ± 0.25*	24.9 ± 0.75*	0.08 ± 0.009	0.8 ± 0.14*	8.3 ± 0.56*	0.09 ± 0.017
24	0.8 ± 0.06*	6.9 ± 0.11*	0.11 ± 0.007*	0.7 ± 0.07*	8.0 ± 0.70*	0.09 ± 0.016
120	1.1 ± 0.15*	6.4 ± 0.87*	0.17 ± 0.008*	1.0 ± 0.28*	7.6 ± 0.10*	0.13 ± 0.025

Results are the mean ± SD

* $P < 0.01$ versus untreated cells by one-way ANOVA and post hoc comparisons by Tukey's test

study [5], reported toxicities in patients due to the accumulation of AG2034 in tissues and further development was discontinued. Nevertheless, AG2034 is an excellent inhibitor of GARFT and the further development of such drugs as therapeutic agents requires a more complete understanding of their biological and metabolic interactions. Since little is known about the effects of such drugs on prostate cancer cells whose energy metabolism is unique, and for which effective chemotherapeutic regimens are essentially nonexistent, we investigated the effects of AG2034 on two well-known androgen independent human prostate cancer cell lines, DU145 and PC-3.

Continuous exposure of DU145 and PC-3 cells to 50 nM AG2034 prevented growth of both cell lines for approximately 7 days at which time, in the absence of hypoxanthine, cell numbers began to decrease indicating cell death (Fig. 2). However, in the presence of 1.7 μM hypoxanthine, which approximates its concentration in human serum, DU145 cells resumed growth after 14 days while the number of PC-3 cells did not change. The fact that PC-3 cells remained adherent to the surface, and were readily able to convert [^{3}H]-hypoxanthine into ATP at day 21 (Table 2) suggests that the continued presence of drug rendered them cytostatic. However, the clonogenic assays shown in Fig. 3 confirm that in the absence of hypoxanthine, AG2034 can be cytotoxic to both cell lines but the drug's effectiveness is substantially inhibited by its presence. This is reminiscent of earlier studies, which reported that 96 h continuous exposure of WiDR colon carcinoma cells to the GARFT inhibitor 5,10-dideazatetrahydrofolate (DDATHF) resulted in retention of adherence of the cells to the surface indicating cytostasis [26]. However, further studies by these authors showed that the drug was cytotoxic as indicated by their inability to clone in the presence of micromolar levels of hypoxanthine [26]. Although the ability of higher concentrations of hypoxanthine to inhibit the effects of GARFT inhibitors is well known [12], the observation that prostate cancer cell lines display resistance to the drug at low physiological levels of this compound, may indicate that these cells are more efficient in utilizing salvage and adapting to blockage of de novo purine synthesis than other cell types. The results in Table 2 show that in the absence of drug, both of these cell lines are able to synthesize ATP via salvage and the results in Fig. 6 indicate that in the presence of drug this level of synthesis is sufficient to maintain similar levels of ATP in both cell lines from 14 to 35 days. It has been reported that in rat hepatoma cells the purine salvage pathway has a higher capacity than the de novo pathway and that in the presence of drugs targeted against the de novo pathway, this capacity increases [28]. In the prostate cancer cell lines used here, salvage synthesis cannot restore the intracellular levels of ATP to those found in the absence of drug (Fig. 6). Whether this is related to the dysregulation of purine biosynthetic pathways known to be associated with prostate cancer [23] remains to be determined. It should be noted that contrary to previous studies using the GARFT inhibitor LY309887 against a variety of solid tumor cell lines [19], ATP depletion alone in DU145 and PC-3 cells treated with AG2034 is not, as indicated in Figs. 2, 3 and 6, a predictor of growth inhibition or cytotoxicity in these androgen independent tumor cell lines.

Table 2 Effect of AG2034 on the conversion of [^{3}H]–HPX into ATP with the continuous treatment of DU145 and PC-3 cells in FA-Plus media over 21 days

AG2034 treatment time (days)	[^{3}H]–HPX converted into ATP (pmol/mg protein)	
	DU145	PC-3
0	7.979 ± 0.484	2.731 ± 0.042
1	1.745 ± 0.258*	1.068 ± 0.117*
14	3.398 ± 0.267*	4.202 ± 0.515*
21	2.393 ± 0.227*	5.338 ± 1.379*

Values denote mean (pmol/mg protein) ± SD

*$P < 0.01$ versus untreated cells by one-way ANOVA and post hoc comparisons by Tukey's test

The ability of DU145 cells to begin growth after 14 days of continuous exposure to drug in the presence of 1.7 μM hypoxanthine at a concentration, 50 nM, that prior to that time effectively prevented proliferation, was unexpected. Since the 14 day time frame during which there is little or no cell division is unlikely to allow for the selection of mutants, the growth of DU145 cells at day 14 might result from the outgrowth of a pre-existing subpopulation of cells able to cope with lowered ATP levels or perhaps might be due to epigenetic alterations associated with the energy imbalance imposed by inhibition of the de novo pathway. Future studies with DU145 cells cloned from the post day-14 population will assist in clarifying these issues. Additionally it is clear that PC-3 cells cannot proliferate under these conditions even though their steady-state levels of ATP post day-14 are similar to DU145 (Fig. 6) and de novo synthesis remains effectively blocked (Fig. 7). This strongly suggests that it is not the cellular concentration of ATP per se that is responsible for the proliferation of DU145 or for the non-proliferation of PC-3 cells under these conditions. Neither does it appear to be a difference in the ability of these cell lines to utilize salvage since both are readily capable of incorporating radiolabeled hypoxanthine into ATP (Table 2). Indeed after 21 days of drug exposure PC-3 cells incorporate more than twice as much hypoxanthine into ATP as DU145 cells (Table 2).

Given that PC-3 cells post day-14 contain an equivalent steady-state amount of ATP as DU145 cells, we considered the possibility that they might be unable to activate the

AMPK signaling pathway. This pathway is sensitive to the AMP/ATP ratio in cells and under conditions of ATP depletion caused by metabolic stress induces the phosphorylation of AMPK which in turn activates several biochemical processes that increase ATP production and inactivates others that consume ATP [16, 18]. In the absence of hypoxanthine, drug-induced depletion of ATP causes an increase in the AMP/ATP ratio in both cell lines but much less so in PC-3 (Table 1). The minimal if any increase in AMPK phosphorylation that follows in PC-3 cells compared with the much greater increase seen in DU145 (Fig. 8) is consistent with a reduced capacity of PC-3 cells to activate the pathway. However, it was reported by Xiang et.al. [29] that PC-3 cells are able to activate AMPK after treatment with AICAR, which is converted by cellular metabolism to ZMP an AMP analog [13]. Since activation of AMPK requires the binding of AMP to its catalytic α subunit [16], the inability of PC-3 cells to activate AMPK may be the result of lowering AMP levels to a point where it can no longer effectively bind AMPK (Table 1). Why DU145 cells can activate AMPK under these same conditions but not in the presence of hypoxanthine (Fig. 8) is unknown. It should be noted here and throughout the report that in addition to 1.7 μM hypoxanthine, FA-Plus medium also contains 1.5 μM thymidine. While the studies presented here focus on the effects of hypoxanthine as a moderator of cellular response to AG2034 treatment, the potential effects that salvageable thymidine may have on our observations remain to be determined.

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