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ORIGINAL ARTICLE

Caffeine markedly sensitizes human mesothelioma cell lines to pemetrexed

Sang Hee Min · I. David Goldman · Rongbao Zhao

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Abstract Pemetrexed is a new generation antifolate approved for the treatment of mesothelioma and non-small cell lung cancer. Caffeine is known to augment radiation or chemotherapeutic drug-induced cell killing. The current study addresses the impact of caffeine on the activity of pemetrexed in mesothelioma cell lines. Caffeine enhanced pemetrexed activity in all four mesothelioma cell lines tested (H2052, H2373, H28 and MSTO-211H). Caffeine sensitized H2052 cells in a dose- and schedule-dependent manner, and was associated with a markedly decreased clonogenic survival. Caffeine sensitization occurred only in cells subjected to pulse, but not continuous, exposure to pemetrexed. Similar pemetrexed sensitization was also observed with the clinically better tolerated caffeine analog, theobromine. Pemetrexed sensitization by caffeine was associated with an increase in pemetrexed-induced phosphorylation of ataxia-telangiectasia-mutated (ATM) and Chk1. These data indicate that caffeine and its analog, theobromine, may be a useful approach to enhance pemetrexed-based chemotherapy.

Keywords Pemetrexed · Caffeine · Theobromine · Mesothelioma

S. H. Min · I. D. Goldman · R. Zhao
Departments of Medicine and Molecular Pharmacology,
Albert Einstein College of Medicine,
Bronx, NY 10461, USA

R. Zhao (✉)
Chanin 628, 1300 Morris Park Ave, Bronx,
NY 10461, USA
e-mail: rzhao@aecom.yu.edu

Introduction

Pemetrexed (alimta®) is a new generation antifolate with thymidylate synthase as its primary, and glycinamide ribonucleotide formyltransferase as its secondary, target [1]. These are key enzymes required for de novo biosynthesis of thymidine and purine nucleotides. Pemetrexed activity is unlikely to be related to an inhibitory effect at dihydrofolate reductase because (1) it is a very weak inhibitor of this enzyme (three orders of magnitude less than that of methotrexate), (2) inhibition of thymidylate synthase blocks dihydrofolate production obviating the need for this enzyme and, (3) thymidine alone protects cells from pemetrexed at drug concentrations that markedly suppress tumor cell growth [2]. Pemetrexed, in combination with cisplatin, has been used as front-line treatment of mesothelioma, heretofore considered chemo-resistant [3]. Pemetrexed, alone, is also approved for second-line treatment of non-small-cell lung cancer based upon a recent phase III trial [4]. This drug is currently being evaluated in other cancers, alone or in combination with other agents (http://www.cancer.gov).

Caffeine at sub- or millimolar concentrations, not toxic to cells, enhances the cytotoxic effect of ionizing radiation. This has been proposed to be based upon an inhibitory effect on ATM/ATR [5–8]. Inhibition of ATM/ATR results in abrogation of the G2 cell-cycle check point and cells treated with caffeine undergo mitosis before DNA is repaired [7, 8]. However, AT cells in which ATM is mutated are still sensitized by caffeine, despite the check point defect. This suggests that caffeine effects cannot be attributed solely to ATM inhibition [9]. Sensitization may also be due to caffeine suppression of homologous recombination-mediated DNA-repair, which is independent of cell cycle signaling, but may accompany caffeine inhibition of ATM [10, 11]. Despite extensive studies on caffeine

radiosensitization, caffeine has rarely been used in combination with radiotherapy clinically because of the extent of side effects associated with sensitizing doses.

Caffeine also sensitizes cancer cells to chemotherapeutics such as nitrogen mustard and thiotepa [12, 13]. Cisplatin sensitization, however, is erratic [14–17]. Caffeine has been reported to desensitize cells to other DNA-damaging drugs such as mitoxantrone, doxorubicin, camptothecin and topotecan. This has been attributed to formation of complexes between caffeine and these aromatic drugs, which reduces their effective concentrations [18, 19]. Caffeine has been included in multi-drug regimens for the treatment of malignant melanoma [20] and pancreatic cancer [20–23], but enhanced activity has not been documented. However, in other studies the addition of caffeine increased response rates in patients with osteosarcoma, high-grade soft tissue sarcomas, metastatic carcinoma and lymphoma [24–26].

In the current study the effect of caffeine on pemetrexed-induced cell growth inhibition and cell death was assessed in mesothelioma cell lines. In addition, the effect of theobromine, a clinically more tolerable caffeine analog, on pemetrexed activity was compared to that of caffeine. A pulse exposure to pemetrexed was chosen to simulate the pharmacokinetics of this drug in clinical regimens. With a standard pemetrexed dose of 500 mg/m^2, the plasma concentration initially reaches more than 200 μM but rapidly decreases to 8 μM after 8 h and to 0.2 μM after 24 h [27]. The effects of caffeine on ATM autophosphorylation and on phosphorylation of the ATR substrate, Chk1, were also assessed. The results suggest a potential role for agents with caffeine-like actions to augment pemetrexed activity in mesothelioma and other neoplasms.

Materials and methods

Chemicals and reagents

Pemetrexed was provided by the Eli Lilly Company (Indianapolis, IN); caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine) and anti-β-actin antibody (clone AC-15, #5441) were obtained from Sigma (St Louis, MO). Antibodies against human phospho-ATM (Serine1981) (#4526) and human phospho-Chk1 (serine317) (#2344) as well as HRP-linked anti-mouse and anti-rabbit IgG were purchased from Cell Signaling Technology (Danvers, MA).

Cells and media

Human mesothelioma cell lines, H2052, H28, and MSTO 211-H, were purchased from the American Type Culture Collection (Manassas, VA); H2373 cells were obtained from the National Cancer Institute. Cells were maintained in RPMI 1640 medium (HyClone, Logan, UT) supplemented with 10% dialyzed fetal bovine serum (Gemini Bio-Products, Calabasas, CA), 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2.

Cell growth inhibition and clonogenic assay

Three different assays were utilized to determine pemetrexed-induced growth inhibition and cell killing in the presence or absence of caffeine.

Pulse exposure to pemetrexed

Figure 1 illustrates how a typical experiment with a 2 h-exposure to pemetrexed was performed. Cells were seeded in 12-well plates at a density of 50,000 cells/well and allowed to grow overnight. Unless otherwise specified, cells were treated with 30 μM pemetrexed for 2 h in the presence or absence of 1 mM caffeine. Pemetrexed was then removed by washing with serum-free medium three times and cells were grown in drug-free medium or medium containing 1 mM caffeine to complete a 2-day treatment with caffeine. Following this, cells were grown in drug-free medium for an additional 3 days before they were trypsinized and cell counts were determined manually on a hemocytometer. Under these conditions cells that were not treated with any drug reached confluence and only cells that exclude Trypan Blue were scored.

Continuous exposure to pemetrexed

Cells were seeded in 96-well plates (100 μl/well) at a density of 1,000 (5-day-exposure) or 3,000 cells/well (3-day

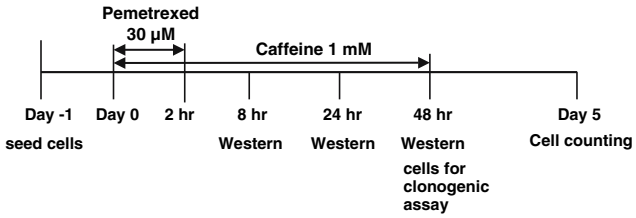

Fig. 1 Schedule of pemetrexed pulse exposure in the presence of caffeine and end points for various measurements. Unless otherwise specified, this figure describes the standard pulse treatment schedule with pemetrexed and caffeine used throughout the studies. Cells were seeded 1 day before they were exposed to pemetrexed and caffeine. Two hours after initiation of pemetrexed (30 μM) and caffeine (1 mM) treatment, cells were washed three times with serum-free medium then grown in drug-free medium or medium containing 1 mM caffeine. Two days after the initiation of pemetrexed exposure, cells were replaced with drug-free medium and allowed to grow for an additional 3 days. For growth inhibition assays, cells were usually counted 5 days after pemetrexed treatment. Western blots analysis was performed 8, 24 or 48 h after initiation of pemetrexed and caffeine exposure. Forty-eight hours after initiation of drug exposure, cells were reseeded for clonogenic assays

exposure) and grown overnight. Cells were then exposed continuously to pemetrexed and caffeine for an additional 3 or 5 days by introducing an equal volume of medium containing different concentrations of pemetrexed and a constant caffeine concentration of 2 or 4 mM. The final concentration of caffeine was either 1 or 2 mM. Cell growth rate was quantified by the sulforhodamine B assay [28].

Clonogenic assay

Cells were exposed to 30 μM pemetrexed for 2 h in the presence or absence of 1 mM caffeine as described above for "pulse exposure to pemetrexed" (see also Fig. 1). After 2 days, cells were trypsinized, counted in Trypan Blue solution, and re-seeded in 6-well plates at a density of 200 or 800 viable cells/well. Fourteen days after plating, colonies were fixed, stained with sulforhodamine B and counted. Clonogenic survival is the ratio (×100) of clones that survived to cells initially seeded.

Western blot

Cells were treated with 30 μM pemetrexed for 2 h in the absence or presence of 1 or 2 mM caffeine as illustrated in Fig. 1. Cells were harvested at 8, 24 and 48 h after initiation of pemetrexed treatment by trypsinization and sonicated (2 to 3-s burst) in a hypotonic buffer (0.5 mM Na_3PO4, 0.1 mM EDTA, pH 7.4). This was followed by centrifugation at 12,000 rpm for 5 min; supernatants were then collected for Western blot analysis. Protein concentrations were determined by BCA Protein Assay (Pierce, Rockford, IL). Equal amounts of protein (~10 μg) were loaded and separated on 12% SDS-PAGE, followed by blotting on a nitrocellulose membrane (Hybond-P, Amersham, Piscataway, NJ). Typically, the membrane was blocked with 5% dry milk in TBS-T (20 mM Tris, 140 mM NaCl, 0.1% Tween-20, pH 7.6) for 1 h and probed with a primary antibody in TBS-T containing 0.5% dry milk for 1 h with a dilution suggested by the manufacturers. The membrane was further incubated with a HRP-linked secondary antibody (1:2000) in TBS-T for 1 h and detected using the Enhanced Chemiluminescence Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire HP7 9NA, UK) and exposed to Kodak Biomax X-ray film (Rochester, NY). For a loading control, the membranes were stripped at 65°C for 30 min in a buffer (62.5 mM Tris, 2% SDS, and 100 mM ß-mercaptoethanol, pH 6.8), and reprobed with an anti-β-actin antibody.

Flow cytometric determination of DNA content

Cells were seeded in 6-well plates at a density of 0.3×10^6 cells/well 1 day before treatment with "pulse exposure to pemetrexed" as described in Fig. 1. Cells attached to, or detached from, plates were collected 8, 24 or 48 h after initiation of pemetrexed treatment. The cells were then combined, washed once with PBS, and fixed with 70% ethanol overnight at 4°C. The cells were pelleted, washed once with PBS and resuspended in 1 ml PBS containing 50 μg/ml propidium iodide and 250 μg/ml RNase, then incubated at room temperature for 30 min. DNA fluorescence signals were acquired and analyzed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) in the Fluorescence Activated Cell Sorting Shared Resource of the Albert Einstein College of Medicine Cancer Center.

Statistical analyses

The data were analyzed by *t* test (One-tailed paired) or ANOVA (Repeated Measures) analyses provided by Graph Pad Prism software (San Diego, CA).

Results

Effect of caffeine on pemetrexed-induced growth inhibition in mesothelioma cell lines

A pulse exposure to pemetrexed was chosen to study pemetrexed-induced growth inhibition to simulate conditions in which the drug is administered in current clinical regimens. Cells were treated with pemetrexed and caffeine as indicated in Fig. 1 except the interval for pemetrexed exposure was varied from 2 to 8 h. In all four mesothelioma cell lines studied, as the duration of exposure to pemetrexed was increased, the extent of growth inhibition increased (Fig. 2). H2052 and H2373 lines were the most sensitive to pemetrexed whereas MSTO-211H cells were the least sensitive among the four cell lines. For example, in the absence of caffeine, incubation with pemetrexed for 8 h reduced cell numbers to 5, 5, 10 or 25% in H2052, H2373, H28 or MSTO-211H cells, respectively. Regardless of the cell line and the pemetrexed exposure time, addition of caffeine during and after pemetrexed exposure significantly reduced cell numbers as compared to pemetrexed treatment alone. The smallest reduction (~50%) was observed with the 2-h-pemetrexed exposure in all cell lines, while the largest reduction (~85%) was found with the 8 h-pemetrexed exposure in MSTO-211H cells. Caffeine at this concentration resulted in only a small (10–20%) reduction in cell numbers in the absence of pemetrexed.

Factors that influence caffeine sensitization

H2052 cells were chosen for further studies to define the factors that influence caffeine sensitization. To determine the onset of caffeine sensitization, H2052 cells were

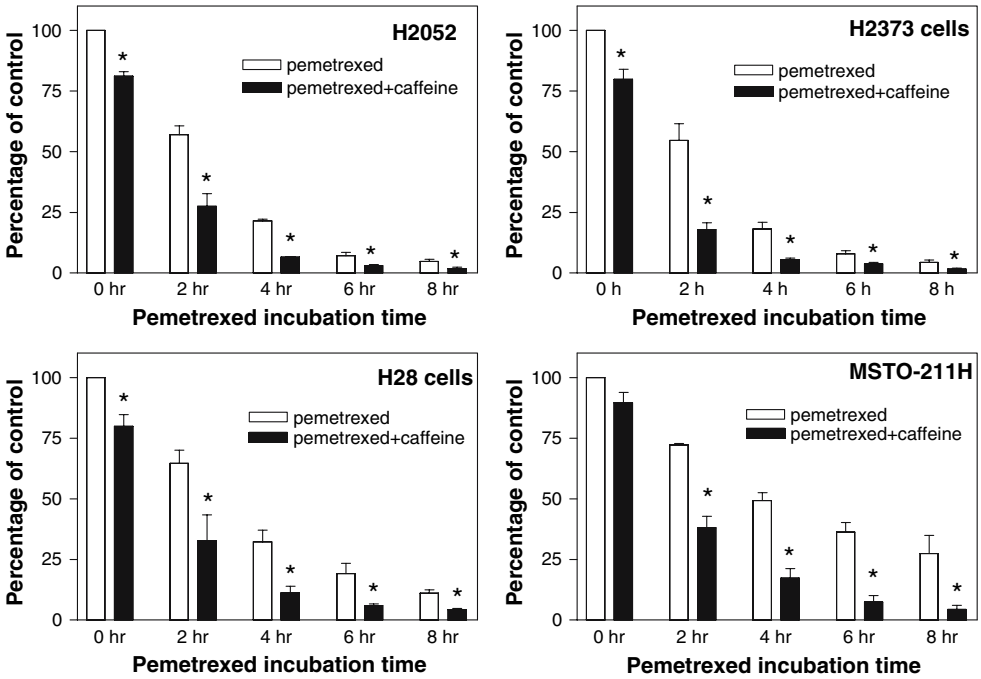

Fig. 2 Caffeine sensitization to pemetrexed in H2052, H2373, H28 and MSTO-211H cells. Cells were exposed to pemetrexed and caffeine as illustrated in Fig. 1 except that the interval of exposure to pemetrexed was varied from 2 to 8 h. The control (0 h) represents the cells not treated with pemetrexed. The data for all three panels are the mean ± SEM from three independent experiments. * The difference is statistically significant ($P < 0.05$) as compared to respective controls (*open bars*)

exposed to pemetrexed for 2 h in the absence or presence of 1 mM caffeine, as illustrated in Fig. 1, and cell numbers were determined every day for up to 7 days. As indicated in Fig. 3a, 1 mM caffeine alone had little effect on cell growth. Exposure of H2052 cells to pemetrexed alone for 2 h delayed cell growth by ~2 days as compared to untreated cells. The combination of 1 mM caffeine and pemetrexed further delayed cell growth by ~2 days. The sensitizing effect of caffeine could only be discriminated 2 days after pemetrexed treatment.

The effect of caffeine on pemetrexed-induced growth inhibition was assessed over a caffeine concentration range from 0.25 to 4 mM. At a concentration less than 2 mM, caffeine alone had only a negligible effect on cell growth; even at 4 mM, caffeine inhibited cell growth only by ~30% (Fig. 3b). However, in the presence of pemetrexed, 1, 2 or 4 mM caffeine decreased cell growth by 72, 92 or 96%, respectively, as compared to cells treated with pemetrexed alone. Sensitization was also detected with caffeine concentrations of 0.25 and 0.5 mM.

The duration of exposure to caffeine required to potentiate pemetrexed activity was also assessed in H2052 cells. As indicated in Fig. 3c, the presence of caffeine in the first 48 hrs after exposure to pemetrexed maximized the synergistic effect. Extending exposure to caffeine to 72 h did not confer additional sensitization. The first 24 h after initiation of pemetrexed treatment appeared to be crucial to the sensitization effect of caffeine.

The effect of caffeine on pemetrexed-induced-growth inhibition with a continuous exposure to pemetrexed was also assessed in H2052 cells. As illustrated in Fig. 3d, neither 1 mM nor 2 mM caffeine had any effect on pemetrexed activity with a 5-day continuous exposure to pemetrexed. Similar results were obtained when cells were exposed continuously to pemetrexed for 3 days (data not shown).

Effect of caffeine on pemetrexed-induced cell killing determined by clonogenic assay

H2052 cells that had been exposed to pemetrexed for 2 h in the absence or presence of 1 mM caffeine were reseeded at the same density 48 h after initiation of pemetrexed treatment (see Figs. 1, 3a) for clonogenic assay. As shown in Fig. 4a and b, 1 mM caffeine alone had no effect on clonogenicity of H2052 cells in the absence of pemetrexed. Likewise, pemetrexed alone did not reduce clonogenicity as compared to untreated cells, indicating that cells were not killed by the brief exposure to this drug. In contrast, clonogenicity in cells treated with both pemetrexed and caffeine was decreased by 80% as compared to untreated cells or cells treated either with pemetrexed alone, or caffeine alone, indicating that caffeine and pemetrexed were highly synergistic. Hence, the addition of caffeine induced a marked decrease in cell viability generated by pemetrexed.

Comparison of the effects of caffeine and theobromine on pemetrexed-induced growth inhibition/cell death

The level of caffeine (1 mM), which was required to markedly sensitize cells to pemetrexed, is not clinically feasible due to its neurological side effects. However, a caffeine analog, theobromine, found in cocoa, is much more clinically

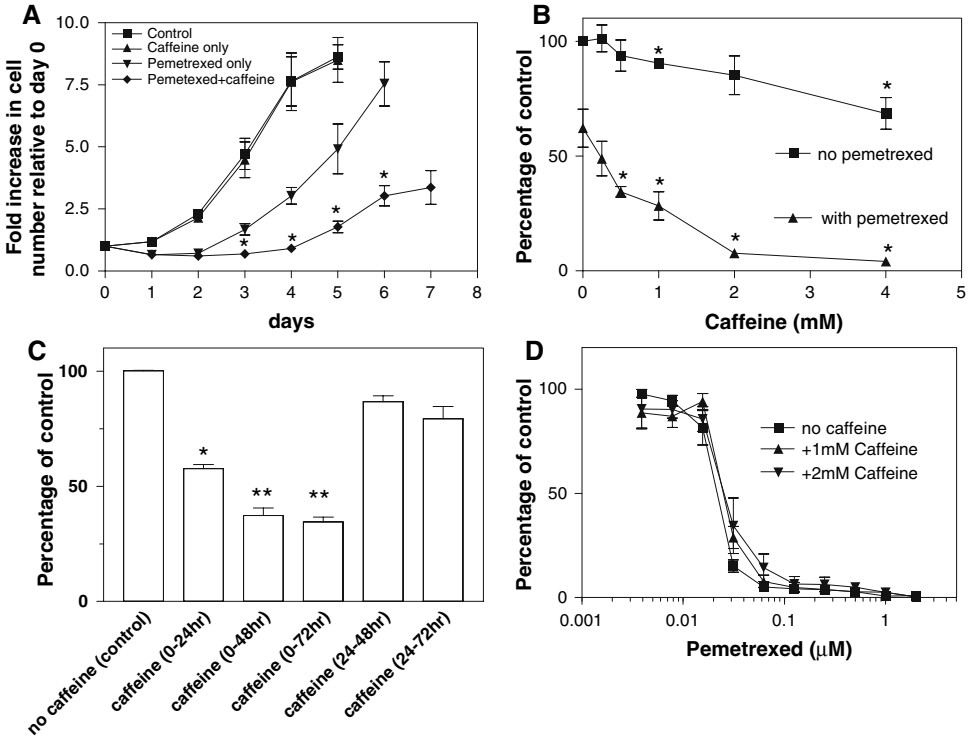

Fig. 3 Factors that influence caffeine sensitization in H2052 cells. **a** H2052 cells were treated with pemetrexed and caffeine as shown in Fig. 1. However, cell numbers were determined each day in the experiment starting from day 0 (initiation of pemetrexed treatment); cell growth is expressed as fold increase in cell numbers as compared to day 0. * The difference is significant ($P < 0.05$) as compared to the "pemetrexed only" group. **b** Treatment of H2052 cells with pemetrexed and caffeine followed the schema in Fig. 1 except that caffeine concentrations were varied from 0.25 to 4 mM. The control represents untreated cells. * The difference is statistically significant ($P < 0.05$) as compared to the respective percentage of control at 0 mM caffeine. **c** Cells were treated with pemetrexed and caffeine as indicated in Fig. 1 except that the period of caffeine incubation was varied. The control represents cells treated with pemetrexed alone. * Indicates significant difference ($P < 0.001$) from the control. ** Indicates a significant difference from the control ($P < 0.001$) and from the "caffeine (0 to 24 h)" group ($P < 0.01$). The difference between them is not statistically significant ($P > 0.05$). **d** H2052 cells were exposed continuously to a spectrum of pemetrexed concentrations and a constant caffeine concentration of either 1 or 2 mM for 5 days. The controls represent cells not exposed to pemetrexed but exposed to the indicated concentrations of caffeine. The data for all panels are the mean ± SEM from three independent experiments

tolerable. For example, a single one-gm dose of theobromine has no adverse effects [29]. Therefore, effects of caffeine (1 mM) and theobromine (1 mM) on pemetrexed activity were compared. As observed for caffeine, theobromine alone had no effect on cell growth ($P > 0.05$) while pemetrexed alone induced ~50% inhibition of growth ($P < 0.001$) (Fig. 5). Theobromine and caffeine produced comparable and marked enhancement of pemetrexed activity, each significantly different from the effects of pemetrexed alone ($P < 0.05$).

Effects of caffeine on pemetrexed-induced phosphorylation of ATM and Chk1

As illustrated in Fig. 6, pemetrexed induced autophosphorylation of ATM at serine 1981; the intensity of phosphorylation 24 or 48 h post-pemetrexed treatment was greater than 8 h post-treatment. Regardless of the duration of exposure, 1 mM caffeine markedly increased activation of ATM. However, when the caffeine concentration was increased from 1 to 2 mM, ATM phosphorylation was decreased to a level comparable to what was observed with pemetrexed alone. The pattern of phosphorylation of Chk1 at serine 317 induced by pemetrexed was quite different. Pemetrexed alone induced Chk1 phosphorylation, but this was gradually decreased as cells recovered after the pemetrexed exposure so that it was barely detectable at 48 h. Addition of caffeine increased phosphorylation of Chk1 in a dose-dependent manner. Thus, 1 mM caffeine increased, rather than inhibited, phosphorylation of ATM and Chk1.

Effect of caffeine on pemetrexed-induced cell-cycle arrest in H2052 cells

The effect of 1 mM caffeine on pemetrexed-induced cell cycle arrest was examined in H2052 cells. As compared to control cells (Fig. 7a), cells treated with caffeine (1 mM) alone had a normal cell-cycle distribution (data not shown).

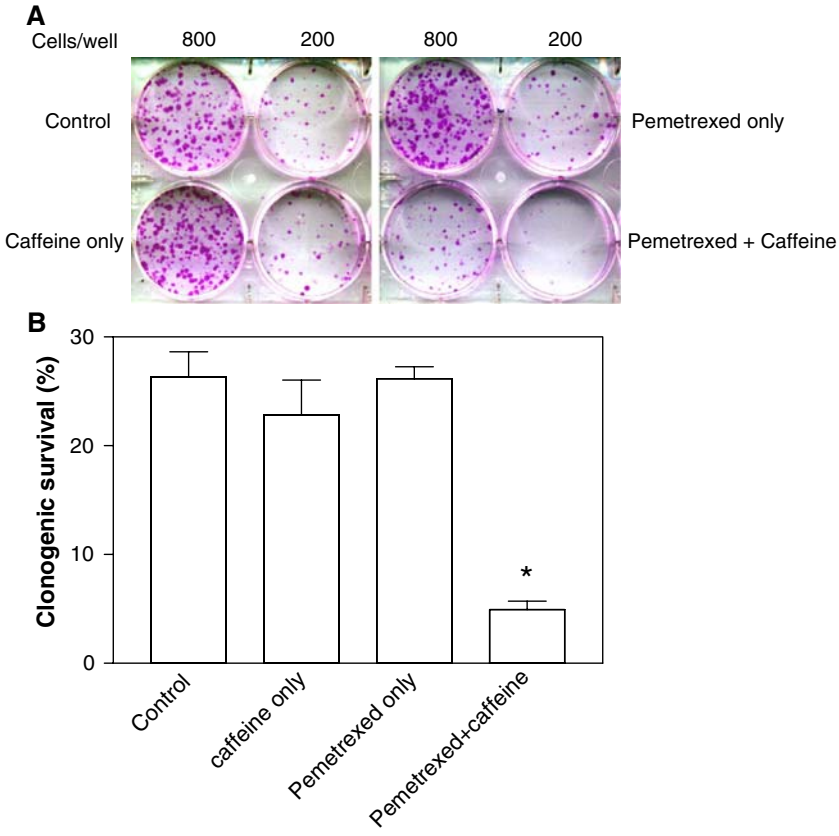

Fig. 4 Effect of caffeine on clonogenic survival of H2052 cells. H2052 cells were exposed to pemetrexed and caffeine as indicated in Fig. 1. After 48 h the cells were trypsinized, counted, and reseeded into 6-well plates at a density of 800 or 200 viable cells/well and were allowed to form colonies in drug-free medium over the following 2 weeks. **a** Represents staining of surviving colonies. The control refers to cells not treated with either drug. **b** Mean ± SEM of three such independent experiments. * Indicates a significant difference ($P < 0.01$) from the other three groups among which there were no significant differences ($P > 0.05$)

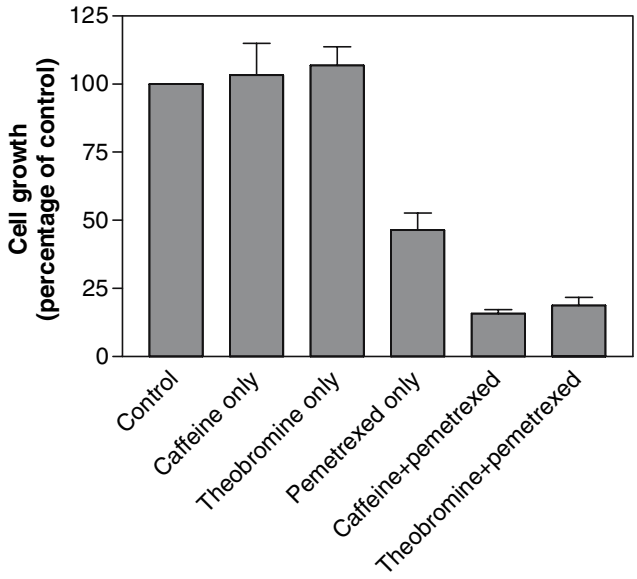

Fig. 5 Comparison of the effects of theobromine and caffeine on pemetrexed activity. H2052 cells were exposed to pemetrexed and 1 mM caffeine or 1 mM theobromine as indicated in Fig. 1. Results are the mean ± SEM of four experiments

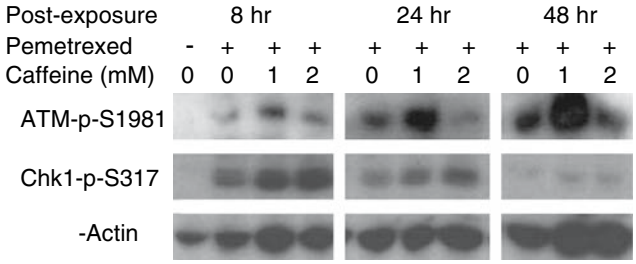

Fig. 6 Western blot analysis of phosphorylation of ATM and Chk1 in H2052 cells exposed to pemetrexed in the presence or absence of caffeine. H2052 cells were exposed to pemetrexed and caffeine as indicated in Fig. 1 with the exception that 2 mM caffeine was also used. Cells were collected by trypsinization and the soluble fractions from cell lysates (~10 μg) were prepared in a hypotonic buffer, separated by 12% SDS-PAGE, blotted onto membranes, and probed with antibodies against ATM-p-S1981 and Chk1-p-S137. Each image is representative of at least two independent experiments. The membrane was stripped and re-probed with an antibody against β-actin for the loading control. Only the β-actin control for Chk1-p-S317 is shown in this panel

When cells were exposed to pemetrexed alone for 8 h, a highly lethal condition, they were arrested at early S-phase, and hardly progressed through S-phase before sub-G1 cells emerged after 48 h (Fig. 7b). However, when cells were treated with pemetrexed alone for only 2 h, a condition that resulted in ~50% growth inhibition and used throughout this study, cells were initially arrested in early S-phase but progressed through S-phase and entered G2/M and G1 after 48 h (Fig. 7c). Inclusion of 1 mM caffeine did not alter the initial S-phase arrest but slightly delayed S-phase progression after 24 or 48 h (Fig. 7d). This effect was more evident

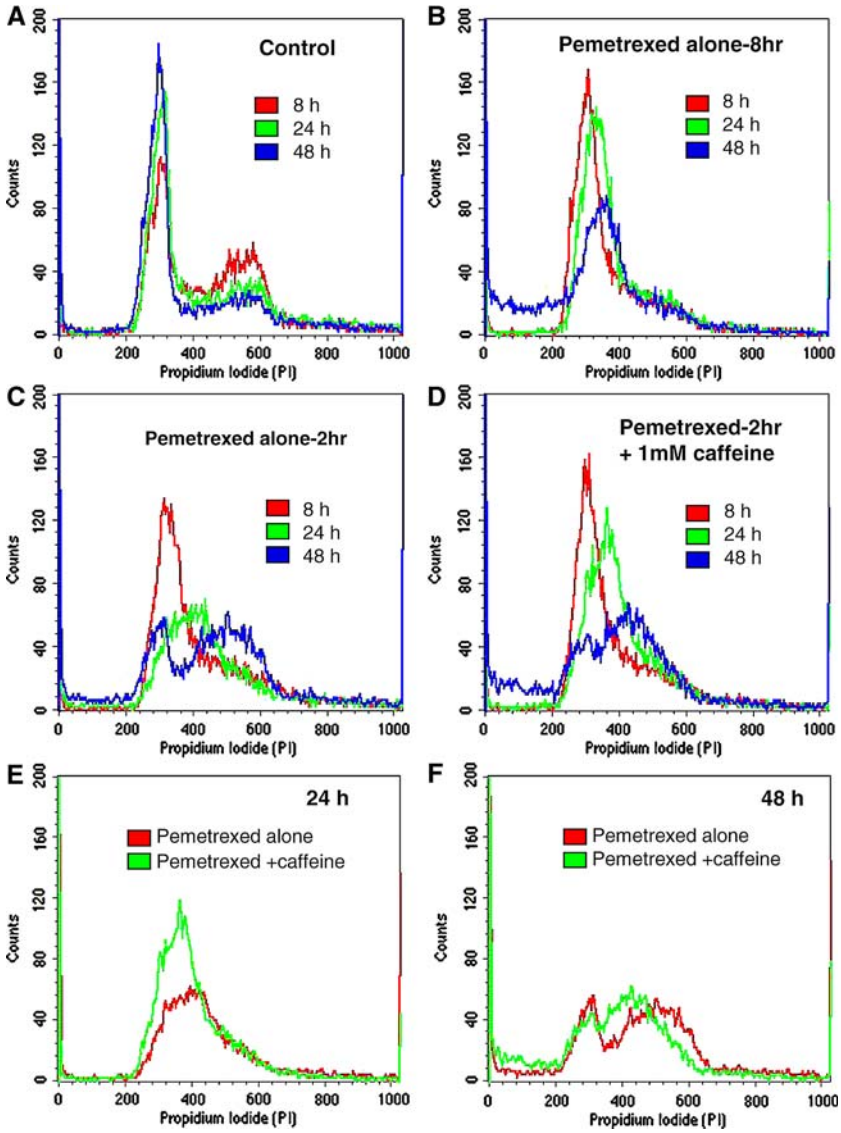

Fig. 7 Impact of caffeine on pemetrexed-induced cell-cycle arrest in H2052 cells. H2052 cells were treated according to the schema in Fig. 1 and collected 8, 24 or 48 h after initiation of exposure to pemetrexed. **a** Control untreated cells; **b** cells exposed to 30 μM pemetrexed for 8 h in the absence of caffeine. Cells exposed to pemetrexed for 2 h in the absence (**c**) or presence (**d**) of 1 mM caffeine. In order to visualize the effect of caffeine more clearly data from (**c**) and (**d**) were re-plotted for cells collected 24 h (**e**) or 48 h (**f**) after initiating pemetrexed treatment. The flow cytometric histograms are representative of three independent experiments

when the data from 24 or 48 h post-pemetrexed treatment were re-plotted in Fig. 7e and f.

Discussion

These studies demonstrate that caffeine markedly sensitizes mesothelioma cells lines to pemetrexed, an effect that only occurs with pulse, and not continuous, exposure to pemetrexed. Hence, the way in which cells are exposed to these drugs will play a critical role in determining the nature of their interaction and whether there will be any interaction at all. The lack of caffeine sensitization when cells were exposed to pemetrexed continuously indicates that caffeine does not alter the interaction between pemetrexed and its target enzymes. The data also raise the possibility that caffeine sensitization of other drugs may have been underestimated if continuous drug exposure, a widely used condition, was employed. Our data further demonstrate that caffeine sensitization of pemetrexed is due to decreased viability of mesothelioma cells that are only growth-arrested but not killed when treated transiently with pemetrexed alone.

There has been a longstanding interest in caffeine as a radio- and chemo-sensitizer, but clinical application of this naturally occurring agent has been largely unexplored. In addition to the lack of incentive for the development of a non-proprietary drug, there have been two major hurdles that have limited the clinical utility of caffeine. (1) There have been conflicting reports on caffeine sensitization of chemotherapeutics in pre-clinical models. (2) The caffeine dose required for sensitization protocols would not be clinically tolerable. The data in the current paper suggest that caffeine sensitization can occur at clinically achievable levels. Administration of 1.5 g/m^2/24 h caffeine intravenously × 3 days has tolerable side effects [26, 30] and plasma caffeine concentrations from 0.2 to 0.4 mM can be sus-

tained [30], levels that sensitize mesothelioma cells to pemetrexed. Our data also raise the possibility that other caffeine analogs that are better tolerated, such as theobromine, may be more feasible, and perhaps more potent, agents to enhance pemetrexed activity.

Caffeine enhancement of pemetrexed activity was prominent in all four mesothelioma cell lines examined despite the fact that all express functional p53 [31]. However, caffeine radiosensitization is less pronounced, or is not observed at all, in cells with functional p53 [32–34]. This discrepancy may be attributed to differences in the effect of p53 on cell-death induced by radiation and pemetrexed. p53-deficient tumor cells are radioresistant [32] while pemetrexed induced cell death is independent of p53 function [35]. Caffeine enhancement of pemetrexed activity in p53-competent cells suggests that it has broader applicability than caffeine-enhanced radiation, since greater than 50% of all tumors lose p53 function. It remains to be determined whether p53-defective cells can be sensitized to pemetrexed by caffeine to a greater extent than p53-intact cells.

Although pemetrexed-induced p53 activation has been reported [36], this is the first report of pemetrexed-induced autophosphorylation of ATM and Chk1, a substrate of ATR. These findings are consistent with the mechanism of action of pemetrexed. Pemetrexed primarily inhibits thymidylate synthase, disrupts DNA synthesis, induces DNA double-strand breaks, and results in phosphorylation of ATM/ATR. However, the increase in pemetrexed-induced phosphorylation of ATM by caffeine is not consistent with the reported inhibitory effect of caffeine on this signal molecule [5–8]. It is possible that caffeine suppression of homologous recombination-mediated DNA-repair [10, 11] results in an increase in double-strand breaks in DNA and, in turn, increases phosphorylation of ATM or ATR (super-activation). This is also consistent with our observations that caffeine delays pemetrexed-induced cell cycle progression. Hence, the effect of caffeine on phosphorylation of ATM depends on two opposing effects, direct inhibition of phosphorylation and enhanced phosphorylation of this molecule due to an increase in DNA double-strand breaks. Super-activation of ATM or ATR substrates, Chk1 and Chk2, in cells treated with genotoxic agents and caffeine has also been reported [37]. However, the possibility that the interaction between caffeine and pemetrexed may involve other targets cannot be excluded.

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References

1. Shih C, Chen VJ, Gossett LS, Gates SB, MacKellar WC, Habeck LL, Shackelford KA, Mendelsohn LG, Soose DJ, Patel VF, Andis SL, Bewley JR, Rayl EA, Moroson BA, Beardsley GP, Kohler W, Ratnam M, Schultz RM (1997) LY231514, a pyrrolo[2,3-d]pyrimidine-based antifolate that inhibits multiple folate-requiring enzymes. Cancer Res 57:1116–1123
2. Zhao R, Zhang S, Hanscom M, Chattopadhyay S, Goldman ID (2005) Loss of reduced folate carrier function and folate depletion result in enhanced pemetrexed inhibition of purine synthesis. Clin Cancer Res 11:1294–1301
3. Vogelzang NJ, Rusthoven JJ, Symanowski J, Denham C, Kaukel E, Ruffie P, Gatzemeier U, Boyer M, Emri S, Manegold C, Niyikiza C, Paoletti P (2003) Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. J Clin Oncol 21:2636–2644
4. Hanna N, Shepherd FA, Fossella FV, Pereira JR, De Marinis F, Von Pawel J, Gatzemeier U, Tsao TC, Pless M, Muller T, Lim HL, Desch C, Szondy K, Gervais R, Shaharyar, Manegold C, Paul S, Paoletti P, Einhorn L, Bunn PA Jr (2004) Randomized phase III trial of pemetrexed versus docetaxel in patients with non-small-cell lung cancer previously treated with chemotherapy. J Clin Oncol 22:1589–1597
5. Sarkaria JN, Busby EC, Tibbetts RS, Roos P, Taya Y, Karnitz LM, Abraham RT (1999) Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. Cancer Res 59:4375–4382
6. Hall-Jackson CA, Cross DA, Morrice N, Smythe C (1999) ATR is a caffeine-sensitive, DNA-activated protein kinase with a substrate specificity distinct from DNA-PK. Oncogene 18:6707–6713
7. Blasina A, Price BD, Turenne GA, McGowan CH (1999) Caffeine inhibits the checkpoint kinase ATM. Curr Biol 9:1135–1138
8. Zhou BB, Chaturvedi P, Spring K, Scott SP, Johanson RA, Mishra R, Mattern MR, Winkler JD, Khanna KK (2000) Caffeine abolishes the mammalian G(2)/M DNA damage checkpoint by inhibiting ataxia-telangiectasia-mutated kinase activity. J Biol Chem 275:10342–10348
9. Asaad NA, Zeng ZC, Guan J, Thacker J, Iliakis G (2000) Homologous recombination as a potential target for caffeine radiosensitization in mammalian cells: reduced caffeine radiosensitization in XRCC2 and XRCC3 mutants. Oncogene 19:5788–5800
10. Wang H, Boecker W, Wang H, Wang X, Guan J, Thompson LH, Nickoloff JA, Iliakis G (2004) Caffeine inhibits homology-directed repair of I-SceI-induced DNA double-strand breaks. Oncogene 23:824–834
11. Golding SE, Rosenberg E, Khalil A, McEwen A, Holmes M, Neill S, Povirk LF, Valerie K (2004) Double strand break repair by homologous recombination is regulated by cell cycle-independent signaling via ATM in human glioma cells. J Biol Chem 279:15402–15410
12. Byfield JE, Murnane J, Ward JF, Calabro-Jones P, Lynch M, Kulhanian F (1981) Mice, men, mustard and methylated xanthines: the potential role of caffeine and related drugs in the sensitization of human tumours to alkylating agents. Br J Cancer 43:669–683
13. Fingert HJ, Chang JD, Pardee AB (1986) Cytotoxic, cell cycle, and chromosomal effects of methylxanthines in human tumor cells treated with alkylating agents. Cancer Res 46:2463–2467
14. Janss AJ, Levow C, Bernhard EJ, Muschel RJ, McKenna WG, Sutton L, Phillips PC (1998) Caffeine and staurosporine enhance the cytotoxicity of cisplatin and camptothecin in human brain tumor cell lines. Exp Cell Res 243:29–38
15. Takahashi M, Yanoma S, Yamamoto Y, Rino Y, Amano T, Imada T (1998) Combined effect of CDDP and caffeine against human gastric cell line in vivo. Anticancer Res 18:4399–4401
16. Boike GM, Petru E, Sevin BU, Averette HE, Chou TC, Penalver M, Donato D, Schiano M, Hilsenbeck SG, Perras J (1990) Chemical enhancement of cisplatin cytotoxicity in a human ovarian and cervical cancer cell line. Gynecol Oncol 38:315–322
17. Deplanque G, Ceraline J, Lapouge G, Dufour P, Bergerat JP, Klein-Soyer C (2004) Conflicting effects of caffeine on apoptosis

and clonogenic survival of human K1 thyroid carcinoma cell lines with different p53 status after exposure to cisplatin or UVc irradiation. Biochem Biophys Res Commun 314:1100–1106
18. Traganos F, Kapuscinski J, Darzynkiewicz Z (1991) Caffeine modulates the effects of DNA-intercalating drugs in vitro: a flow cytometric and spectrophotometric analysis of caffeine interaction with novantrone, doxorubicin, ellipticine, and the doxorubicin analogue AD198. Cancer Res 51:3682–3689
19. Traganos F, Kapuscinski J, Gong J, Ardelt B, Darzynkiewicz RJ, Darzynkiewicz Z (1993) Caffeine prevents apoptosis and cell cycle effects induced by camptothecin or topotecan in HL-60 cells. Cancer Res 53:4613–4618
20. Cohen MH, Schoenfeld D, Wolter J (1980) Randomized trial of chlorpromazine, caffeine, and methyl-CCNU in disseminated melanoma. Cancer Treat Rep 64:151–153
21. Dougherty JB, Kelsen D, Kemeny N, Magill G, Botet J, Niedzwiecki D (1989) Advanced pancreatic cancer: a phase I-II trial of cisplatin, high-dose cytarabine, and caffeine. J Natl Cancer Inst 81:1735–1738
22. Al Sukhun S, Zalupski MM, Ben Josef E, Vaitkevicius VK, Philip PA, Soulen R, Weaver D, Adsay V, Heilbrun LK, Levin K, Forman JD, Shields AF (2003) Chemoradiotherapy in the treatment of regional pancreatic carcinoma: a phase II study. Am J Clin Oncol 26:543–549
23. Ahmed S, Vaitkevicius VK, Zalupski MM, Du W, Arlauskas P, Gordon C, Kellogg C, Shields AF (2000) Cisplatin, cytarabine, caffeine, and continuously infused 5-fluorouracil (PACE) in the treatment of advanced pancreatic carcinoma: a phase II study. Am J Clin Oncol 23:420–424
24. Tsuchiya H, Tomita K, Mori Y, Asada N, Morinaga T, Kitano S, Yamamoto N (1998) Caffeine-assisted chemotherapy and minimized tumor excision for nonmetastatic osteosarcoma. Anticancer Res 18:657–666
25. Tsuchiya H, Tomita K, Yamamoto N, Mori Y, Asada N (1998) Caffeine-potentiated chemotherapy and conservative surgery for high-grade soft-tissue sarcoma. Anticancer Res 18:3651–3656
26. Hayashi M, Tsuchiya H, Yamamoto N, Karita M, Shirai T, Nishida H, Takeuchi A, Tomita K (2005) Caffeine-potentiated chemotherapy for metastatic carcinoma and lymphoma of bone and soft tissue. Anticancer Res 25:2399–2405
27. Latz JE, Chaudhary A, Ghosh A, Johnson RD (2006) Population pharmacokinetic analysis of ten phase II clinical trials of pemetrexed in cancer patients. Cancer Chemother Pharmacol 57:401–411
28. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82:1107–1112
29. Usmani OS, Belvisi MG, Patel HJ, Crispino N, Birrell MA, Korbonits M, Korbonits D, Barnes PJ (2005) Theobromine inhibits sensory nerve activation and cough. FASEB J 19:231–233
30. Kawahara M, Kagiyama H, Kanazawa Y, Tsuchiya H, Tomita K, Yokogawa K, Miyamoto K (2004) Rapid determination method of caffeine and application to monitoring of caffeine-assisted chemotherapy. Biopharm Drug Dispos 25:61–67
31. Manfredi JJ, Dong J, Liu WJ, Resnick-Silverman L, Qiao R, Chahinian P, Saric M, Gibbs AR, Phillips JI, Murray J, Axten CW, Nolan RP, Aaronson SA (2005) Evidence against a role for SV40 in human mesothelioma. Cancer Res 65:2602–2609
32. Powell SN, DeFrank JS, Connell P, Eogan M, Preffer F, Dombkowski D, Tang W, Friend S (1995) Differential sensitivity of p53(−) and p53(+) cells to caffeine-induced radiosensitization and override of G2 delay. Cancer Res 55:1643–1648
33. Russell KJ, Wiens LW, Demers GW, Galloway DA, Plon SE, Groudine M (1995) Abrogation of the G2 checkpoint results in differential radiosensitization of G1 checkpoint-deficient and G1 checkpoint-competent cells. Cancer Res 55:1639–1642
34. Yao SL, Akhtar AJ, McKenna KA, Bedi GC, Sidransky D, Mabry M, Ravi R, Collector MI, Jones RJ, Sharkis SJ, Fuchs EJ, Bedi A (1996) Selective radiosensitization of p53-deficient cells by caffeine-mediated activation of p34cdc2 kinase. Nat Med 2:1140–1143
35. Lu X, Errington J, Curtin NJ, Lunec J, Newell DR (2001) The impact of p53 status on cellular sensitivity to antifolate drugs. Clin Cancer Res 7:2114–2123
36. Longley DB, Boyer J, Allen WL, Latif T, Ferguson PR, Maxwell PJ, McDermott U, Lynch M, Harkin DP, Johnston PG (2002) The role of thymidylate synthase induction in modulating p53-regulated gene expression in response to 5-fluorouracil and antifolates. Cancer Res 62:2644–2649
37. Cortez D (2003) Caffeine inhibits checkpoint responses without inhibiting the ataxia-telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR) protein kinases. J Biol Chem 278:37139–37145