行政院國家科學委員會專題研究計畫 成果報告

反轉肺腺癌細胞對多重抗葉酸類抗癌藥 Pemetrexed(愛寧達)之抗藥性的可行性

研究成果報告(精簡版)

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計 畫 主 持 人 ： 許國堂
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公 開 資 訊 ： 本計畫可公開查詢

中 華 民 國 101 年 10 月 03 日

中 文 摘 要 ： Pemetrexed (愛寧達，Alimta)為葉酸拮抗劑抗癌藥物，作用機轉是分別抑制 glycinamide ribonucleotide formyltransferase (GARFT)，dihydrofolate reductase (DHFR)與 thymidylate synthase (TS)三個酶，而使其作用更廣泛。這些酵素蛋白是癌細胞製造合成 DNA 及 RNA 所需 thymidine 及 purine nucleotides 的關鍵步驟，因此可以藉此作用來殺死癌細胞。愛寧達似乎是對非小細胞肺癌中的非鱗狀上皮癌(non-squmous types)有較佳的療效，因此被推薦用來治療非鱗狀上皮類之肺癌。因為其治療肺癌的效果和一線藥物相同，因此自 2009 年二月起，衛生署已經核准愛寧達可用於肺腺癌的第一線化療用藥，讓肺癌病患在化療第一時間就可以選用愛寧達，不必像過去，得等到第一線化療失敗後才能使用。可是目前對於愛寧達之抗藥性產生之機轉並無深入的研究，基本上都是以舊一代的單一作用之葉酸拮抗劑抗癌藥物產生之抗藥性做為論點，這和愛寧達是多重作用之抗葉酸藥物的本質不符。因此，研究肺癌對愛寧達之抗藥性產生之機轉就顯得非常需要。

為此，我們原先提出三年的研究計畫包含下列兩個目標：壹、建立愛寧達的抗藥性肺腺癌細胞株進行基因體分析，找出主要的抗藥基因並且使用干擾 RNA 的技術抑制其表現或將其過度表達來觀察其與抗藥性產生之關聯。貳、探討是否可以使用臨床使用之化療用藥做為當愛寧達治療抗藥性產生後時之第二線藥物，並且使用小鼠模式來驗證其可適用性。因為此計畫只有得到壹年的經費補助 (601,000 元)，因此，我們與徐士蘭教授進行協同合作來完成可以達成的標的。已經發表壹篇期刊論文(Pemetrexed induces both intrinsic and extrinsic apoptosis through ataxia telangiectasia mutated/p53-dependent and -independent signaling pathways.) Yang TY, Chang GC, Chen KC, Hung HW, Hsu KH, Wu CH, Sheu GT, Hsu SL. Mol Carcinog. 2011 Nov 15. [Epub ahead of print]. IF: 3.265 (ONCOLOGY 63/184, 34.2%). 尚有部份數據正在補充，希望可以再發發表壹篇期刊論文。

中文關鍵詞： 肺腺癌，抗葉酸類抗癌藥，愛寧達，抗藥性

英 文 摘 要 ： Pemetrexed is a novel multitargeted antifolate that inhibits one or several key folate-requiring enzymes of the thymidine and purine biosynthetic pathways, in particular, thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT). Previous studies have identified a plethora of mechanisms of antifolate resistance frequently associated with alterations in influx and/or efflux transporters of (anti)folates as well as in folate-dependent enzymes. These include inactivating mutations and/or down- regulation of the reduced folate carrier (RFC), DHFR, TS and folylpolyglutamate synthase (FPGS). Whether all of above mentioned alterations would result in pemetrexed-resistance is not well demonstrated. Therefore, a clear characterization of the mechanisms to overcome pemetrexed-resistance is required. Originally, we have proposed to characterize the drug resistance and reversal of resistance of pemetrexed in human lung adenocarcinoma in a three-year interval as following: (1) the first goal is to establish pemetrexed-resistant cells and analyzed the differential expressed genes. (2) The second goal is to compare and analyze the suitable chemotherapeutic drugs as second line therapy for patient whom has permetrexed refractory. The sensitive and resistant genes for permetrexed treatment will be determined. Because we only have funded for one year with total of NT $601,000, therefore, we started a collaboration with Prof. Hsu, who was also interested in pemetrexed regulated pathways, to complete our goal in this proposal. We have published one article (Pemetrexed induces both intrinsic and extrinsic apoptosis through ataxia telangiectasia mutated/p53-dependent and -independent signaling pathways.) Yang TY, Chang GC, Chen KC, Hung HW, Hsu KH, Wu CH, Sheu GT, Hsu SL. Mol Carcinog. 2011 Nov 15. [Epub ahead of print]. With IF:3.265 (ONCOLOGY 63/184, 34.2%). There are more data under collection for one more manuscript for submission.

英文關鍵詞： lung adenocarcinoma, antifolate, drug resistance, pemetrexed

行政院國家科學委員會補助專題研究計畫	□期中進度報告
	x 期末報告

（計畫名稱）

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本計畫除繳交成果報告外，另含下列出國報告，共 ___ 份：
□移地研究心得報告
□出席國際學術會議心得報告
□國際合作研究計畫國外研究報告

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中　華　民　國　101 年　10　月 03　日

中文摘要及關鍵詞 **(keywords)**：**肺腺癌，抗葉酸類抗癌藥，愛寧達，抗藥性**

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報告內容：

Introduction:

Part 1. Molecular basis of pemetrexed resistance

♦ Antametabolites inhibition of 5-FU, methotrexate (MTX), and pemetrexed

Antimetabolites have been major therapeutic agents for the treatment of cancer for many years. Antimetabolites in clinical practice include the inhibitors of thymidylate synthase (TS) and dihydrofolate reductase (DHFR) (Jackman & Calvert, 1995; Rustum et al, 1997). TS is a critical enzyme in DNA synthesis because it is rate-limiting in the production of thymidine nucleotides, which are required exclusively for DNA synthesis. For many years, the fluoropyrimidine 5-fluorouracil (5-FU) has been the most frequently used TS inhibitor. 5-FU remains a major agent in the treatment of breast cancer, colon cancer, and many other malignancies. DHFR inhibitors such as methotrexate (MTX), trimetrexate, edatrexate, and others are all folate analogues. MTX has activity in a variety of cancers, including hematologic malignancies and breast, lung, and head and neck cancers. The development of new folate-based TS and DHFR inhibitors has been hampered by the occurrence of unexpected severe and sometimes lethal toxicities, including stomatitis, neutropenia, and sepsis (Calvert & Bunn, 2002).

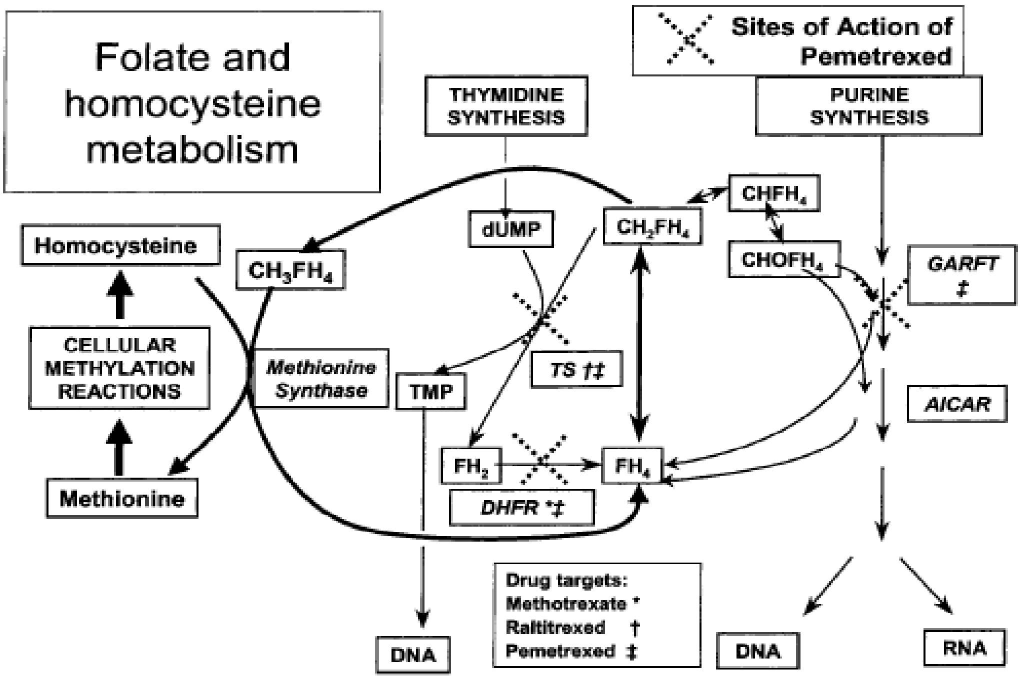

Fig 1. A diagram depicting the sites of action of various antifolate drugs and the interaction of folate and homocysteine metabolism. Note that methionine synthase is vitamin B_{12} dependent. Any reduction in the activity of methionine synthase caused by poor availability of intracellular folate or vitamin B_{12} deficiency will increase homocysteine levels because of a reduction in the rate of conversion of homocysteine to methionine. FH_2, dihydrofolate; FH_4, tetrahydrofolate; $CHFH_4$, methenyltetrahydrofolate; CH_2FH_4, methylenetetrahydrofolate; CH_3FH_4, methyltetrahydrofolate; DHFR, dihydrofolate reductase; TS, thymidylate synthase; GARFT, glycinamide ribonucleotide formyltransferase; AICAR, aminoimidazolcarboxamide ribosyltransferase.

♦ Mechanisms of pemetrexed action.

Pemetrexed (Alimta, LY231514, Eli Lilly and Co, Indianapolis, IN) was developed because it inhibits at least three key enzymes involved in DNA synthesis including TS, DHFR, and glycinamide ribonucleotide formyltransferase (GARFT), as shown in Fig 1 (Calvert & Bunn, 2002). Pemetrexed enters cells via the reduced folate carrier (RFC), with transport kinetics similar to that of MTX, and binds to folate receptor-α with a very high affinity, similar to that of folic acid (Zhao et al, 2000). Pemetrexed also appears to be a substrate for multidrug resistance protein transporters (Wielinga et al, 2005; Zeng et al, 2001). Intracellularly, pemetrexed is polyglutamated to the active pentaglutamide by a reaction catalyzed by folylpolyglutamate synthase (FPGS). Pemetrexed is one of the best substrates for FPGS when compared to other antifolates such as MTX (Goldman & Zhao, 2002). Pemetrexed can be considered a prodrug, because its pentaglutamate form is the predominant intracellular form, and is over 60-fold more potent in its inhibition of TS than the parent compound (Shih et al, 1998). Polyglutamation traps pemetrexed and enhances its intracellular retention. The parent drug is polyglutamated 90- to 200-fold more efficiently than MTX and 6- to 13-fold more efficiently than the GARFT inhibitor, lometrexol (Shih et al, 1997). The increased cellular retention of polyglutamated pemetrexed forms may explain the success of the 3-week administration schedule. Pemetrexed inhibits multiple enzyme targets involved in both pyrimidine and purine synthesis. One of these primary enzyme targets is TS, a folate-dependent enzyme, catalyzes the transformation of dUMP to dTMP. Inhibition of TS results in decreased thymidine necessary for DNA synthesis (Schultz et al, 1999). In addition to TS, pemetrexed inhibits DHFR, aminoimidazole carboxamide ribonucleotide formyltransferase, as well as GARFT; the latter is a folate-dependent enzyme that is involved in purine synthesis (Shih et al, 1997). These targets are related to the cytotoxicity of pemetrexed, because both thymidine and hypoxanthine are required to circumvent cellular death caused by pemetrexed (Shih et al, 1997). Pemetrexed is 30–200 times more potent an inhibitor of TS than of aminoimidazole carboxamide ribonucleotide formyltransferase or GARFT, suggesting that its cytotoxicity may be mediated predominantly through TS inhibition.

Mechanisms of antifolates resistance overview

The antifolates were the first class of antimetabolites to enter the clinics more than 50 years ago. Over the following decades, a full understanding of their mechanisms of action and chemotherapeutic potential evolved along with the mechanisms by which cells develop resistance to these drugs. These principals served as a basis for the subsequent exploration and understanding of the mechanisms of resistance to pemetrexed. This section describes the bases for intrinsic and acquired antifolate resistance within the context of the current understanding of the mechanisms of actions and cytotoxic determinants of these antifolates. This encompasses (1) impaired drug transport into cells, augmented drug export, (2) impaired activation of antifolates through polyglutamylation, augmented hydrolysis of antifolate polyglutamates, (3) increased expression and mutation of target enzymes.

Alterations in TS

(Overexpression of TS) Acquired resistance to antifolates that target TS has been associated with increased expression or, in a few cases, mutations in this enzyme that alter drug binding. Overexpression of TS is an important mechanism of resistance in small lung cancer cell lines selected in the presence of pemetrexed (Ozasa et al, 2009). As observed with DHFR, this is frequently associated with gene amplification. For example, resistance to ZD1694 in human lymphoblastoid (O'Connor et al, 1992), and ovarian carcinoma cell lines (Freemantle et al, 1995) were associated with TS amplification. The relationship between the increase in expression and the level of resistance can vary. Hence, while 20,000-fold resistance in human lymphoblastoid cells was associated with a 1,000-fold increase in the TS protein level, 14-fold resistance in ovarian carcinoma cells was associated with only a 2.5-fold increase in TS activity. Recently, Ozasa *et al*. (Ozasa et al, 2010) established pemetrexed-resistant small cell lung cancer cell lines to investigate the mechanisms of acquired resistance to pemetrexed. They found the TS gene expression was significantly increased in resistant cells. Knockdown of TS expression using siRNA enhanced pemetrexed cytotoxicity in PC6 ⁄ MTA-4.0 cells. Their results suggested that up-regulation of the expression of the TS gene may have an important role in the acquired resistance to pemetrexed. In addition, TS may be a predictive marker for pemetrexed sensitivity in lung cancer.

The first goal of this project was to determine the major genes associated with pemetrexed resistance. We will examine the RFC, ABCC, ABCG, FPGS, GGH, DHFR, TS and GARFT expression and possible mutations.

Part 2. Reversal of pemetrexed resistance with other chemotherapeutic drugs

We directly examined the response of A549/A400 subline, a permetrexed- resistant lung cancer cell line, with different chemotheraputic drugs and found several interesting data for further investigation. The first novel finding is A549/A400 subline is more sensitive to vincristine than parental A549 cells. This is a very surprising data that indicate permetrexed resistance could be relived by another chemotheraputic drug. Another finding is that A549/A400 subline, only developed a cross-resistance with gemcitabine (Gemzar) and MTX but not to docetaxel and 5-FU. These data indicate the sequential use of a chemotheraputic drug affect efficacy of pemetrexed treatment. A recent article have mentioned that in NSCLC, prior gemcitabine-base treatment has higher objective response rate and progression-free survivals with subsequent pemetrexed therapy (Sun et al, 2009). Another article also reported that sequential administration of pemetrexed followed by docetaxel may provide the greatest anti-tumor effects for lung cancer treatment (Kano et al, 2009). Recently, Wu *et al*. have shown downregulation of TS and DHFR genes and upregulation of p21, p27, Lcn-2, and nm23-H1 genes may serve as new biomarkers for predicting responsiveness to pemetrexed (Wu et al, 2010). **Therefore, we proposed to examine how vincristine re-sensitize the pemetrexed-resistant cell lines *in vitro* and *in vivo* with A549/A400 subline.**

Significance of this project: The sensitive and resistant genes for pemetrexed treatment will be determined. Therefore, those genes can be applied clinically to develop new strategies for lung cancer patients who are suitable to use pemetrexed as first-line chemotherapy. Also, the patients who developed pemetrexed resistance can have better opportunity to overcome drug resistance with follow up treatment.

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已出版的論文：

Pemetrexed Induces Both Intrinsic and Extrinsic Apoptosis Through Ataxia Telangiectasia Mutated/p53-Dependent and -Independent Signaling Pathways

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Pemetrexed, a new-generation antifolate, has demonstrated promising single-agent activity in front- and second-line treatments of non-small cell lung cancer. However, the molecular mechanism of pemetrexed-mediated antitumor activity remains unclear. The current study shows that pemetrexed induced DNA damage and caspase-2, -3, -8, and -9 activation in A549 cells and that treatment with caspase inhibitors significantly abolished cell death, suggesting a caspase-dependent apoptotic mechanism. The molecular events of pemetrexed-mediated apoptosis was associated with the activation of ataxia telangiectasia mutated (ATM)/p53-dependent and -independent signaling pathways, which promoted intrinsic and extrinsic apoptosis by upregulating Bax, PUMA, Fas, DR4, and DR5 and activating the caspase signaling cascade. Supplementation with dTTP allowed normal S-phase progression and rescued apoptotic death in response to pemetrexed. Overall, our findings reveal that the decrease of thymidylate synthase and the increase of Bax, PUMA, Fas, DR4, and DR5 genes may serve as biomarkers for predicting responsiveness to pemetrexed. © 2011 Wiley Periodicals, Inc.

Key words: apoptosis; ataxia telangiectasia mutated; p53; pemetrexed; PUMA

INTRODUCTION

Lung cancer is the leading cause of cancer death for men and women in most industrialized countries, and by 2008, there were an estimated 1.61 million new cases, representing 12.7% of all new cancers [1]. Non-small cell lung cancer accounts for approximately 80–85% of cancer cases [2,3]. Most patients with non-small cell lung cancer have locally advanced or metastatic disease at the time of diagnosis, and a large proportion of patients who initially present with the early stage of non-small cell lung cancer ultimately relapse with metastatic disease and require systemic treatments [3]. Cisplatinum-based combination chemotherapy is the standard front-line treatment for patients with advanced non-small cell lung cancer. The median survival time and 1-yr survival rate are only approximately 8 mo and 33%, respectively [4].

Pemetrexed is a multiple-targeted antifolate cytotoxic agent, which potently inhibits thymidylate synthase (TS), glycinamide ribonucleotide formyltransferase, and dihydrofolate reductase [5]. In the past decade, pemetrexed has had an increasingly established role in the treatment of patients with advanced non-small cell lung cancer, especially adenocarcinoma. The phase III trials have shown that pemetrexed has equal efficacy and favorable toxicity and safety profiles compared to previously standard cytotoxic drugs in first- and second-line

Abbreviations: TS, thymidylate synthase; KU, KU55933; PFT, Pifithrin-α; CA, caffeine; ATM, ataxia telangiectasia mutated; TRAIL, TNF-related apoptosis-inducing ligand; H2AX, histone 2AX.

The authors declare that they have no conflict of interest.

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treatment [6,7]. Clinical trials have demonstrated the survival benefit of pemetrexed as a maintenance therapy following cisplatin-based doublet chemotherapy in advanced non-small cell lung cancer [8]. Undoubtedly, pemetrexed will be extensively used in non-small cell lung cancer patients. Previous studies have shown that antifolates cause mature and nascent DNA double strand breaks in human non-small cell lung cancer A549 cells [9] and in human colon cancer cells [10]. Cell cycle kinetics indicates that S-phase arrest is essential for the induction of DNA damage. However, relatively little is known about the events leading from strand breaks to cell death after pemetrexed treatment. The aim of the current study was to examine the main pharmacological aspects and the responsible mechanisms of pemetrexed activity in human non-small cell lung cancer cells. This is the first report that describes the involvement of ataxia telangiectasia mutated (ATM) and p53-dependent and -independent consequences in pemetrexed-induced growth arrest and apoptosis in human non-small cell lung cancer cells.

MATERIALS AND METHODS

Reagents

Pemetrexed was dissolved in sterile water. KU55933 (KU) was purchased from Calbiochem (VWR International AB, Stockholm, Sweden). Pifithrin-α (PFT) and caffeine (CA) were purchased from Sigma Co. (St. Louis, MO). Anti-Fas and anti-Fas ligand antibodies were purchased from BD Biosciences Pharmingen (San Diego, CA). Anti-phosphorylated $p53^{Ser15}$ was purchased from Cell Signaling Technology (Danvers, MA). Anti-β-actin, anti-Bax, anti-Bcl-2, anti-$p21^{CIP/WAF1}$, anti-$p27^{KIP1}$, and anti-p53 antibodies and ATM siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-TNF-related apoptosis-inducing ligand (TRAIL) receptor 1 (DR4), anti-TRAIL receptor 2 (DR5), and anti-phosphorylated $ATM^{Ser1981}$ antibodies were obtained from Abcam PLC (Cambridge, UK). Anti-$\gamma H2AX^{Ser139}$ antibody was purchased from Upstate Biotechnology (Temecula, CA). Caspase-2, -3, -8, -9, -12 activity assay kits were purchased from R&D Systems (Minneapolis, MN). The caspase-2 inhibitor (Z-VDVAD-FMK), caspase-3 inhibitor (Z-DEVD-FMK), caspase-8 inhibitor (Z-IETD-FMK), and caspase-9 inhibitor (Z-LEHD-FMK) were purchased from Kamiya Biomedical Company (Thousand Oaks, CA). dTTP was obtained from Promega BioSciences, Inc (San Luis Obispo, CA).

Cell Culture

The human non-small cell lung cancer A549 (wild-type p53) and H1299 (p53 null) cell lines were obtained from American Type Culture Collection (ATCC; Manassas, VA) and cultured in RPMI 1640 supplemented with 5% fetal bovine serum, 2 mM glutamine, and antibiotics (100 unit/mL penicillin and 100 μg/mL streptomycin), at 37°C in a 5% CO_2 humidified atmosphere. The culture medium was changed every 2 d. A stable clone expressing p53 *sh*RNA in A549 cells, named A549-*sh*p53 was established as previously described [11].

Comet Assay

Cells were incubated with or without 1 μM pemetrexed for 16, 24, and 48 h, and then embedded in 1.5% of low-melting agarose and spread on microscope slides which had covered with 1% normal-melting agarose. The cells were lysed (lysis buffer: 5 M NaCl, 100 mM EDTA, 100 mM Tris, 1% Triton-X 100) for 10 min on ice. After lysis, the cells were immersed in alkaline solution (0.3 M NaOH, 1 mM EDTA, pH 13) for 10 min on ice and electrophoresed at 300 mA and 25 V for 25 min to unwind the DNA. The slides were neutralized in buffer (0.4 M Tris–HCl, pH 7.5) for 5 min before staining with a solution with 20 μg/mL ethidium bromide in phosphate-buffered saline (PBS). Ethidium bromide-stained nuclei were observed and photographed using a fluorescence microscope. Images of a minimum of 100 cells per treatment were analyzed using the CometScore software (Tritek Corporation, Sumerduck, VA). The tail moment [% DNA in tail × tail length (μm)] was used as parameter to evaluate DNA damage.

Protein Preparation and Western Blot Analysis

The cells were cultured with or without pemetrexed for the indicated times. After treatment, both adherent and floating cells were harvested, washed twice with ice-cold PBS and lysed in ice-cold modified RIPA buffer. After 30 min of incubation on ice, the cells were centrifuged at 100 000*g* for 30 min at 4°C, and the supernatants were collected. The protein concentration was determined using the Bradford method. For Western blot analysis, equal amounts of total protein were loaded onto SDS–polyacrylamide gels and the proteins electrophoretically transferred onto a PVDF membrane (Millipore, Bedford, MA). Immunoblots were analyzed using specific primary antibodies. After probing with horseradish peroxidase-conjugated secondary antibody for 1 h, proteins were visualized using an enhanced chemiluminescence detection kit (ECL Kits; Amersham Life Science).

TUNEL Assay for Apoptotic Cells

The tested cells were treated with pemetrexed for the indicated time periods, washed with PBS twice, fixed in 2% paraformaldehyde for 20 min and then permeabilized with 0.1% Triton X-100/PBS for 30 min at room temperature. After washing with PBS, terminal transferase-mediated dUTP-fluorescensin nick end-labeling (TUNEL) assay was performed according to the manufacturer's instructions (Boehringer, Mannheim, Germany). The cells were incubated in TUNEL reaction buffer in a 37°C humidified chamber for 1 h in the dark, then rinsed twice with PBS and incubated with DAPI (1 mg/mL) at 37°C for 10 min. The stained cells were visualized using a fluorescence microscope. The changes in fluorescence were investigated under a fluorescence microscope or analyzed using flow cytometry. TUNEL-positive cells were counted as apoptotic cells.

Caspase Activity Assays

Cells lysates were obtained from 1 μM pemetrexed-treated or untreated cells and tested for caspase-2, -3, -8, -9, and -12 activities by the addition of the fluorogenic peptide substrate (100 μM) for caspase-2 (VDVAD-AFC), caspase-3 (DEVD-AFC), caspase-8 (IETD-AFC), caspase-9 (LEHD-AFC), and caspase-12 (ATAD-AFC) conjugated with the fluorescent reporter molecule 7-amino-4-trifluoromethyl coumarin (R&D Systems). The caspase cleaved the fluorogenic peptide and released a fluorochrome that was excited by light at 405 nm and emitted fluorescence at 505 nm. The level of caspase enzymatic activity in the cell lysate was directly proportional to the fluorescence signal that was detected using a fluorescent microplate reader (Fluoroskan Ascent; Labsystems, Helsinki, Finland).

Immunofluoresence Staining

The cells were seeded onto coverslips, treated with or without 1 μM pemetrexed for 48 h and harvested. The cells were washed twice with PBS, incubated with 0.2 μM MitoTracker probe at 37°C for 1 h, and then fixed with 2% paraformaldehyde at room temperature for 20 min. The cells were permeabilized with 0.1% Triton X-100/PBS solution at room temperature for 30 min. After air-drying, the cells were incubated with a monoclonal antibody against Bax at 37°C for 1 h. The coverslips were then washed three times with PBS and detected with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies.

siRNA Transfection

The expression of ATM was knocked down by transient transfection of ATM specific siRNA. A549 cells (2×10^5 cells per well) were seeded in six-well plates overnight. siRNA (25 or 50 nM) was mixed with 6 μL of lipofectamine 2000 (Invitrogen) diluted in antibiotic-free medium. Complex formation was allowed to proceed for 30 min at room temperature prior to adding siRNA dropwise to cells and incubating with cells for 24 h. After treatment, the media were changed to normal growth media containing 1 μM pemetrexed for the indicated times.

Statistical Analysis

The figures presented in the current study were representative of at least three separate experiments with similar pattern. The data were presented as means ± SD from three independent experiments. Statistical differences were evaluated using Student's *t*-test and considered significant at $^{*}P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$.

RESULTS

Pemetrexed Induced DNA Damage and Caspase Activation

TS is a major target of pemetrexed The inhibition of TS reduces dTTP and increases dUTP in cells The continued imbalance in dTTP/dUTP pools is known to cause DNA damage (DNA strand breakage) [9,12] and induces downstream events, leading to apoptosis. To examine the possible DNA damage and DNA strand breaks that are generated in pemetrexed-treated cells, the Comet assay was performed in A549, A549-*sh*p53, and H1299 cells. As shown in Figure 1A (upper panel), a representative micrograph of DNA fluorescently stained DNA in cells showed undamaged and supercoiled DNA remaining within the nuclear cell membrane in control cells. However, pemetrexed-treated cells displayed denatured DNA fragments migrating out from cell in a long comet tail. DNA damage was observed as early as 16 h and reached a maximum at an incubation period of 48 h. The comet tail moments increased in a time-dependent manner in pemetrexed-treated A549, A549-*sh*p53, and H1299 cells (Figure 1A, lower panel).

A good biological correlation exists between DNA damage and cytotoxicity, especially apoptotic cell death, and the activation of the caspase signaling cascade is one of the hallmark features of apoptosis. It is necessary to measure the caspase activity in response to pemetrexed stimuli in this study. As depicted in Figure 1B, treatment with 1 μM pemetrexed significantly increased the activities of caspase-2, -3, -8, and -9 but not casapase-12 compared to controls. Additionally, caspase inhibitors were used to define whether a particular caspase played a crucial role in pemetrexed-induced apoptosis. As depicted in Figure 1C, all of the tested caspase inhibitors (including the caspase-2, -3, -8, and -9 inhibitors) effectively blocked pemetrexed-triggered cell death. These results indicate that both intrinsic and extrinsic caspases were required for pemetrexed-induced cell death.

Pemetrexed triggers both intrinsic and extrinsic apoptosis Bcl-2 family members are evolutionarily conserved and essential mediators in the intrinsic apoptotic pathway in mitochondria. Therefore, the

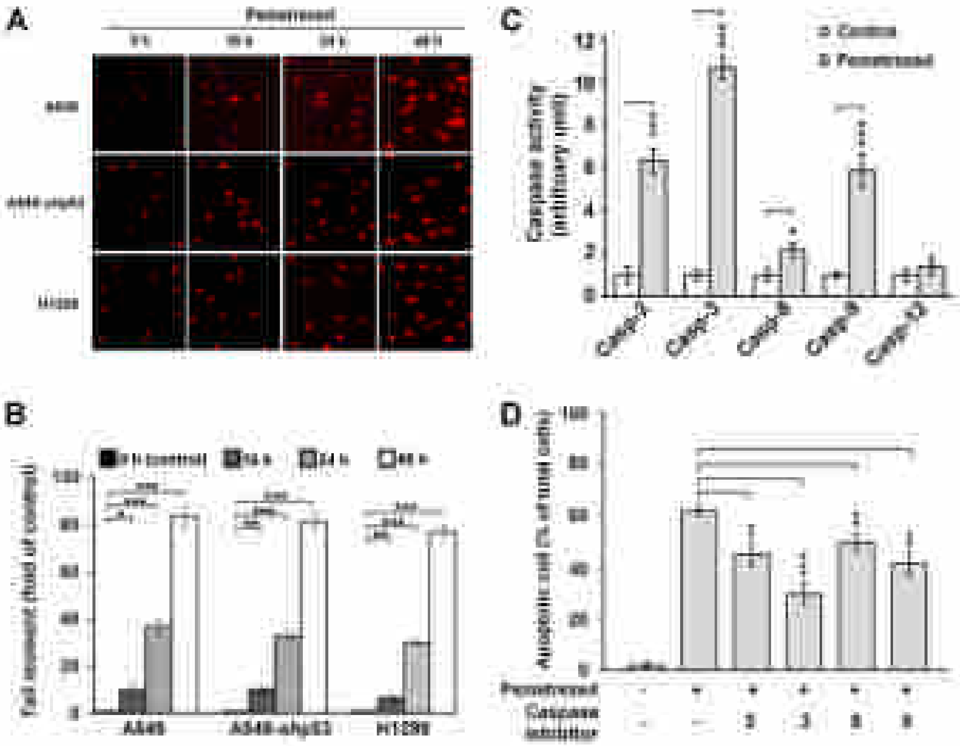

Figure 2. Pemetrexed induces DNA damage, caspase activation, and caspase-dependent apoptosis. (A) DNA damage response. A549, A549-shp53, and H1299 cells were treated with or without 1 μM pemetrexed for indicated time, and DNA damage on per-cell basis was determined using the comet assay. Representative comet images from the cells exposed to pemetrexed at various times were shown (upper panel). The degree of DNA damage was scored by tail moment (% DNA in tail × tail length) from at least 100 cells in each treatment group (lower panel). Data are mean ± SD for three independent experiments. *P < 0.05; ***P < 0.001. (B) The activation of caspases by pemetrexed. Cell lysates were obtained from pemetrexed-treated or untreated cells and tested for caspase-2, -3, -8, -9, and -12 activities by fluorometric caspase assay kits, according to manufacturer's instructions. (C) The caspase inhibitor attenuated pemetrexed-induced apoptosis. A549 cells were incubated with 1 μM of pemetrexed for 72 h, and a TUNEL assay was performed for apoptotic cell determination as described in the Materials and Methods Section.

effect of pemetrexed on the expression and function of Bcl-2 family proteins in A549 cells were examined by Western blot analysis. As indicated in Figure 2A, the level of PUMA was increased by pemetrexed treatment in a dose- and time-dependent manner. Bax levels were induced only with 1 μM pemetrexed treatment for 48 h, whereas the Bcl-2 levels were not affected by pemetrexed administration. Bax is an executor of the mitochondrial pathway of apoptosis, the translocation of Bax from the cytosol to the mitochondria is a critical step of apoptosis [13]. To characterize the subcellular localization of Bax, A549 cells were incubated with or without 1 μM pemetrexed, fixed and subjected to immunofluorescence staining using anti-Bax monoclonal antibody. Mitochondria were stained with MitoTracker Red. In Figure 2B, Bax had a diffuse distribution throughout untreated A549 cells. However, treatment with 1 μM pemetrexed resulted in a cellular redistribution of Bax as shown by immunostaining and confocal imaging. The immunoreactivity of Bax formed a punctate pattern, and the yellow color in the overlay of Figure 2B indicates the colocalization of Bax with mitochondria, indicating the translocation of Bax to mitochondria after pemetrexed treatment. To address the role of Bcl-2 family molecules in pemetrexed-induced apoptosis, A549 cells were transduced with 50 and 100 MOI (multiplicity of infection) of adeno-Bcl-2 and control adenoviral vectors. As shown in Figure 2C, infection with the adeno-Bcl-2 viral vector increased the intracellular Bcl-2 levels and protected against pemetrexed-induced apoptotic death. Next, we analyzed the pemetrexed effect on Bax-knockdown A549 cells which were stably transfected with Bax-specific shRNA as described elsewhere [14]. As depicted in Figure 2D, Bax-knockdown A549 cells showed resistance to pemetrexed-triggered apoptosis.

The activation of cell surface death receptors by tumor necrosis factor superfamily cytokines is a critical regulator of the extrinsic apoptotic pathway. Western blot analysis was performed to explore the effect of pemetrexed on the expression of death receptors in A549 cells. As shown in

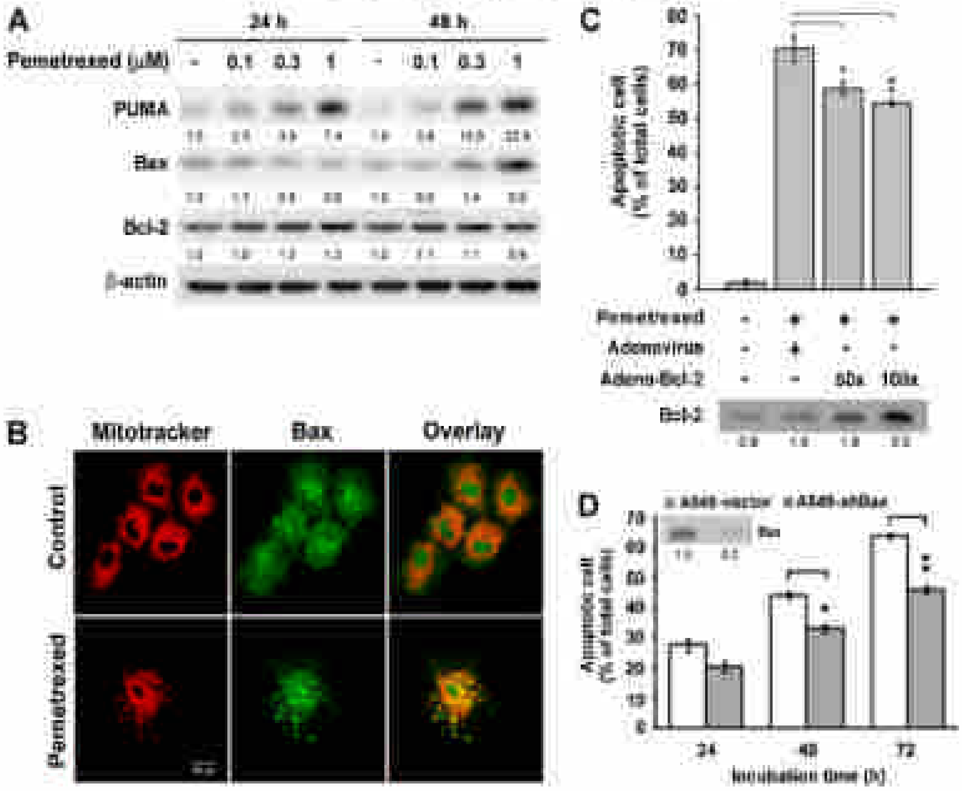

Figure 2. Regulation of Bcl-2 family molecules by pemetrexed. (A) The expression of Bcl-2 family proteins in A549 cells was detected at 24 and 48 h with vary concentrations of pemetrexed. An equal amount of cell lysate was analyzed by Western blotting with β-actin as the loading control. (B) Pemetrexed induced translocation of Bax to mitochondria. A549 cells were treated with or without 1 μM pemetrexed for 48 h, fixed and subjected to immunofluorescence staining using anti-Bax monoclonal antibodies. Mitochondria were stained with MitoTracker Red. (C) Bcl-2 overexpression attenuated pemetrexed-induced apoptosis. A549 cells were infected with the Bcl-2 or lacZ adenoviral vectors at 0, 50, or 100 MOI. One day after infection, cells were treated with 1 μM of pemetrexed for an additional 72 h. Apoptotic cell death was determined by TUNEL assay. Immunoblot was shown for Bcl-2 expression in cells infected with or without the Bcl-2 adenovirus. (D) Knockdown of Bax decreased pemetrexed-induced apoptosis. A549-vector and A549-shBax cells were treated with or without 1 μM pemetrexed for indicated time periods. Apoptotic cell death was determined by TUNEL assay. Immunoblot was shown for Bax expression in cells transfected with or without the Bax shRNA. Results are mean ± SD for three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

Figure 3A, the expressed levels of death receptor molecules, including Fas, Fas-L, DR4, and DR5 were significantly increased after pemetrexed administration. However, the expression levels of tumor necrosis receptors I and II were not altered by pemetrexed (data not shown). In addition, the apoptotic effect of pemetrexed was drastically abolished by Fas neutralizing antibody (Figure 3B) and DR4 antagonist (DR4:Fc) (Figure 3C). These results indicated that both Bcl-2 family- and death receptor-activated signaling pathways contribute to pemetrexed-induced apoptosis.

Pemetrexed Causes p53-Dependent and -Independent Apoptosis

The tumor suppressor p53 is a multifunctional, highly regulated, and promoter-specific transcriptional factor, which is a pivotal component in the cellular response to DNA damage. It is well documented that p53 is phosphorylated and accumulated during DNA damage and plays a critical role in DNA damage-induced apoptosis [15]. Because pemetrexed induces DNA damage in lung cancer cells, the effect of pemetrexed on the expression and function of p53 was examined by Western blot analysis. As expected, the phosphorylation and accumulation of p53 was increased beginning at 24 h postpemetrexed treatment, which was maintained at 48 h (Figure 4A). To determine whether p53 is responsible for pemetrexed-induced apoptosis, we analyzed the effect of pemetrexed in the presence of PFT, a p53 inhibitor [16]. Before pemetrexed treatment, A549 cells were pretreated with PFT (3 or 10 μM) for 2 h. The percentage of pemetrexed-induced apoptotic cells was analyzed using the TUNEL assay at 72 h. The results show that PFT, at a concentration of 10 μM, had no effect on cell viability, although it moderately attenuated pemetrexed-induced apoptosis (Figure 4B). To further confirm whether p53 is responsible for pemetrexed-induced cytotoxicity and to avoid the non-specific effects of PFT, we

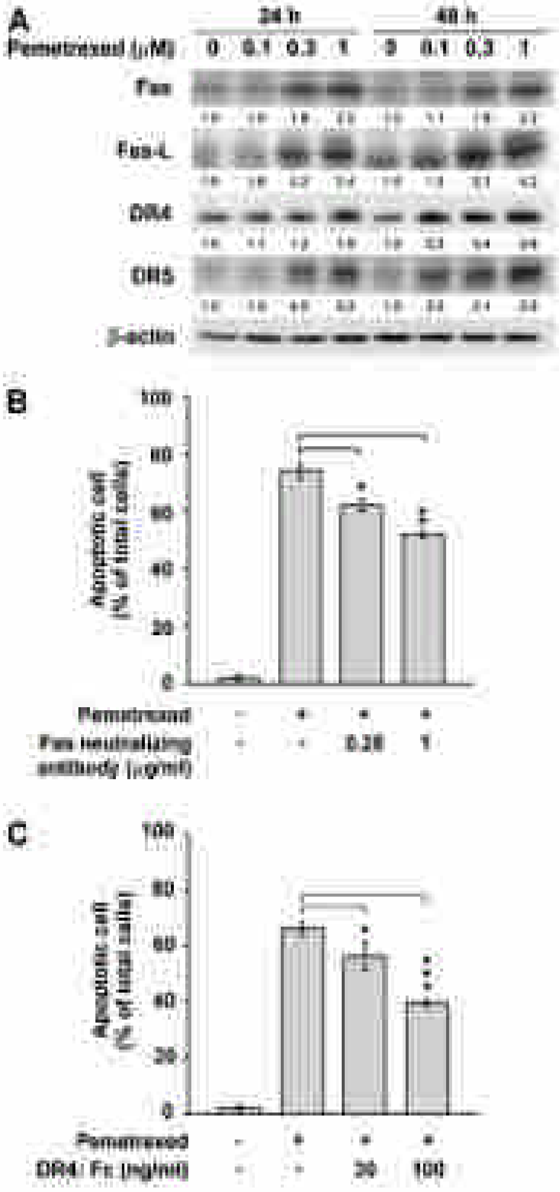

Figure 5. Death receptor signaling is involved in pemetrexed-triggered apoptosis. (A) Western blot analysis demonstrated a time-dependent increase in protein expression of Fas, Fas-L, DR4, and DR5 following pemetrexed treatment of A549 cells. Treatment with anti-Fas neutralizing antibody (B) or DR4/Fc (C) attenuated pemetrexed-induced apoptosis. A549 cells were treated with 1 μM of pemetrexed and anti-Fas antibody or DR4/Fc (an antagonist of DR4 death signaling) at indicated concentrations for 72 h. Apoptotic cell death was determined by TUNEL assay. Results are represented as mean ± SD for three independent experiments. $^{*}P < 0.05$, $^{***}P < 0.001$.

used siRNA silencing to deplete endogenous p53. Figure 4C shows that knockdown of p53 had an only marginal effect on pemetrexed-induced cell death as compared to A549 cells. This event was accompanied by increased PUMA, Bax, Fas, DR4, and DR5 expression (Figure 4D). Therefore, we concluded that p53-dependent and -independent apoptotic pathways were activated following pemetrexed treatment in the tested lung cancer cell lines.

ATM Is One of the Upstream Players of Pemetrexed-Mediated Apoptotic Signaling

DNA damage triggers the activation of ATM by promoting its auto-phosphorylation at Ser1981 [17-19]. H2AX and p53 are the substrates phosphorylated by ATM and are involved in ATM-dependent DNA damage responses [20,21]. We show here that treatment of A549 cells with pemetrexed induces ATM activation and phosphorylation of H2AX at the Ser139 residue (named γH2AX) (Figure 5A), beginning at 24 h of pemetrexed administration. A pronounced and sustained phosphorylation of ATM and γH2AX was observed after 48 h of pemetrexed treatment. There was no effect on the levels of total ATM and H2AX after pemetrexed treatment. The pharmacological inhibitors of ATM, caffeic acid (CA), and KU [22] were used to elucidate whether ATM is involved in the cellular responses to pemetrexed. Pretreatment of cells with CA or KU strongly reduced pemetrexed-induced phosphorylation of both $\gamma H2AX^{Ser139}$ and $p53^{Ser15}$ (Figure 5B) and apoptotic cell death (Figure 5C). In addition, a significant decrease in the levels of PUMA, Bax, Fas, DR4, and DR5 proteins was noticed in cells treated with pemetrexed (Figure 5D). To further address this issue, A549 cells were transfected with ATM siRNA on two successive days to deplete ATM expression prior to pemetrexed treatment. Figure 5E shows that ATM siRNA reduced approximately 70–80% of the ATM expression, which had a profound effect in attenuating pemetrexed-induced $\gamma H2AX^{Ser139}$ and $p53^{Ser15}$ phosphorylation compared to the control siRNA transfection. Moreover, ATM knockdown significantly reduced the levels of Bax, PUMA, Fas, and DR4 proteins (Figure 5E) and apoptosis induced by pemetrexed (Figure 5F). These results suggest that ATM activation plays an upstream role in the pemetrexed-induced $\gamma H2AX^{Ser139}$ and $p53^{Ser15}$ phosphorylation and apoptotic events.

Effects of ATM and p53 on Pemetrexed-Induced S-Phase Cell Cycle Arrest

Our recent report and other studies demonstrated that pemetrexed causes S-phase cell cycle arrest in many types of cancer cells in vitro [23]. To examine whether ATM and p53 activation contribute to pemetrexed-induced S-phase arrest, A549 cells were treated with CA, KU or PFT 2 h prior to pemetrexed treatment. After 24 h of pemetrexed incubation, the cell cycle distribution was analyzed using flow cytometry. As depicted in Figure 6A (upper panel), treatment with pemetrexed led to a significant S-phase arrest by greater than threefold compared with untreated cells.

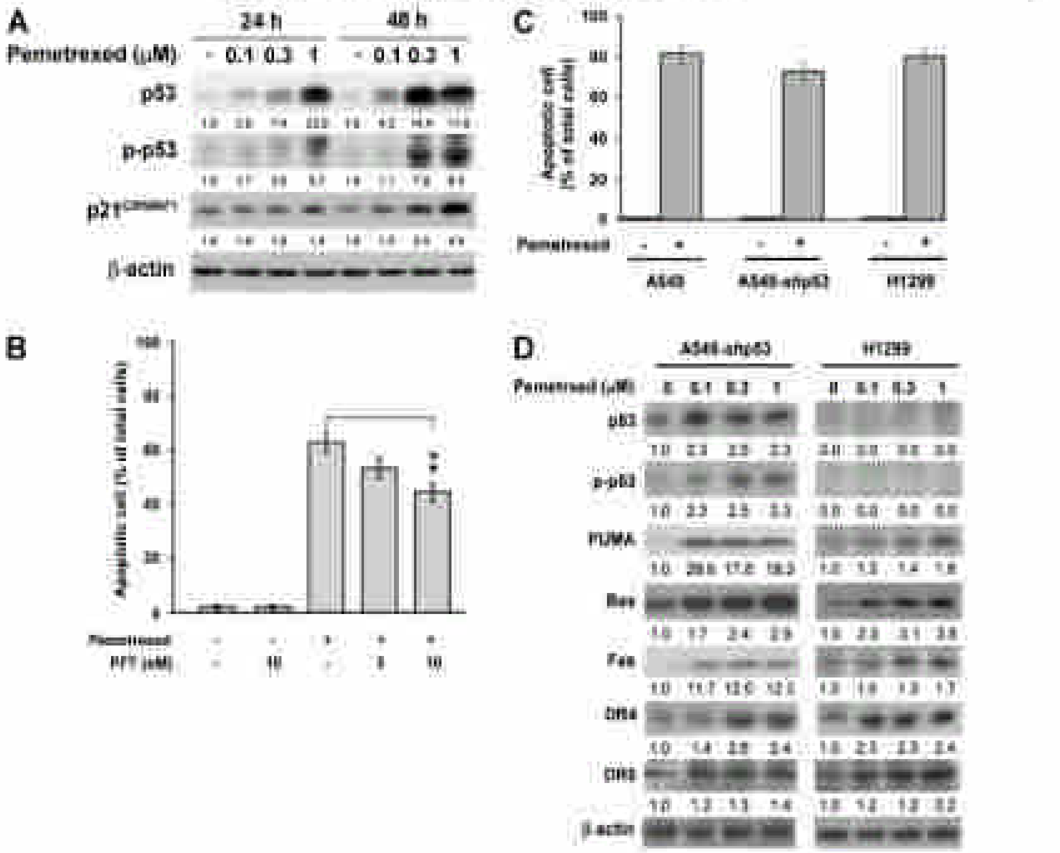

Figure 4. Involvement of p53 in pemetrexed-induced apoptosis. (A) Western blot analysis demonstrated the time- and dose-dependent increases in phosphorylated p53 and $p21^{CIP1/WAF1}$ protein expression following pemetrexed treatment. A549 cells were treated as indicated in Figure 2A, and β-actin was used as loading control. (B) p53 inhibitor attenuated pemetrexed-induced apoptosis. A549 cells were pretreated with indicated concentrations of PFT (pifithrin-α, a p53 inhibitor) for 2 h and treated with or without 1 μM pemetrexed for another 72 h. Apoptotic cell death was determined by TUNEL assay. (C) p53-null H1299 cells and p53-knockdown A549 cells were sensitive to pemetrexed treatment. The tested cells were treated with or without pemetrexed (1 μM) for 72 h, apoptotic cell death was determined by TUNEL assay. (D) Regulation of proapoptotic molecules by pemetrexed. A549-shp53 and H1299 cells were treated as indicated in Figure 2A. Whole-cell lysates were subjected to Western blot analysis using the indicated antibodies. Results are represented as mean ± SD for three independent experiments. $^{**}P < 0.01$.

However, no differences in S-phase population were noted among CA-, KU-, or PFT-treated cells after pemetrexed treatment. The same phenomenon was observed in H1299 cells (Figure 6A, lower panel). Next, A549 (p53 wild), A549-shp53 (p53 knockdown), and H1299 (p53 null) cells were treated with pemetrexed for 24 h, and the cell cycle distribution was examined. As indicated in Figure 6B, there was no significant difference in S-phase population among all tested cell lines. Knockdown of p53 did not induce any observable change in pemetrexed-mediated S-phase arrest. These results indicate that ATM and p53 activation were dispensable for S-phase accumulation caused by pemetrexed.

Exogenous Supplement With dTTP Completely Rescues S-Phase Arrest and Cell Death Induced by Pemetrexed

Since pemetrexed is an inhibitor of TS, it can deplete the dTTP pools in cells, leading to S-phase arrest and apoptosis [9,12]. To confirm this molecular event, exogenous dTTP was added in the absence or presence of pemetrexed in A549 and H1299 cells. As shown in Figure 7A, dTTP alone did not affect the cell cycle progression and cell survival in these tested cell lines. However, the addition of exogenous dTTP in the presence of pemetrexed completely restored the normal S-phase progression as compared with control cultures. Moreover, supplement with dTTP also strongly inhibited pemetrexed-mediated cytotoxicity in tested cells (Figure 7B).

DISCUSSION

Pemetrexed has demonstrated a survival benefit as a maintenance therapy after cisplatin-based doublet chemotherapy in advanced non-small cell lung cancer [8]. Understanding the mechanisms underlying the antitumor properties of pemetrexed is needed for optimization of therapeutic targeting by pemetrexed. We performed a series of studies to

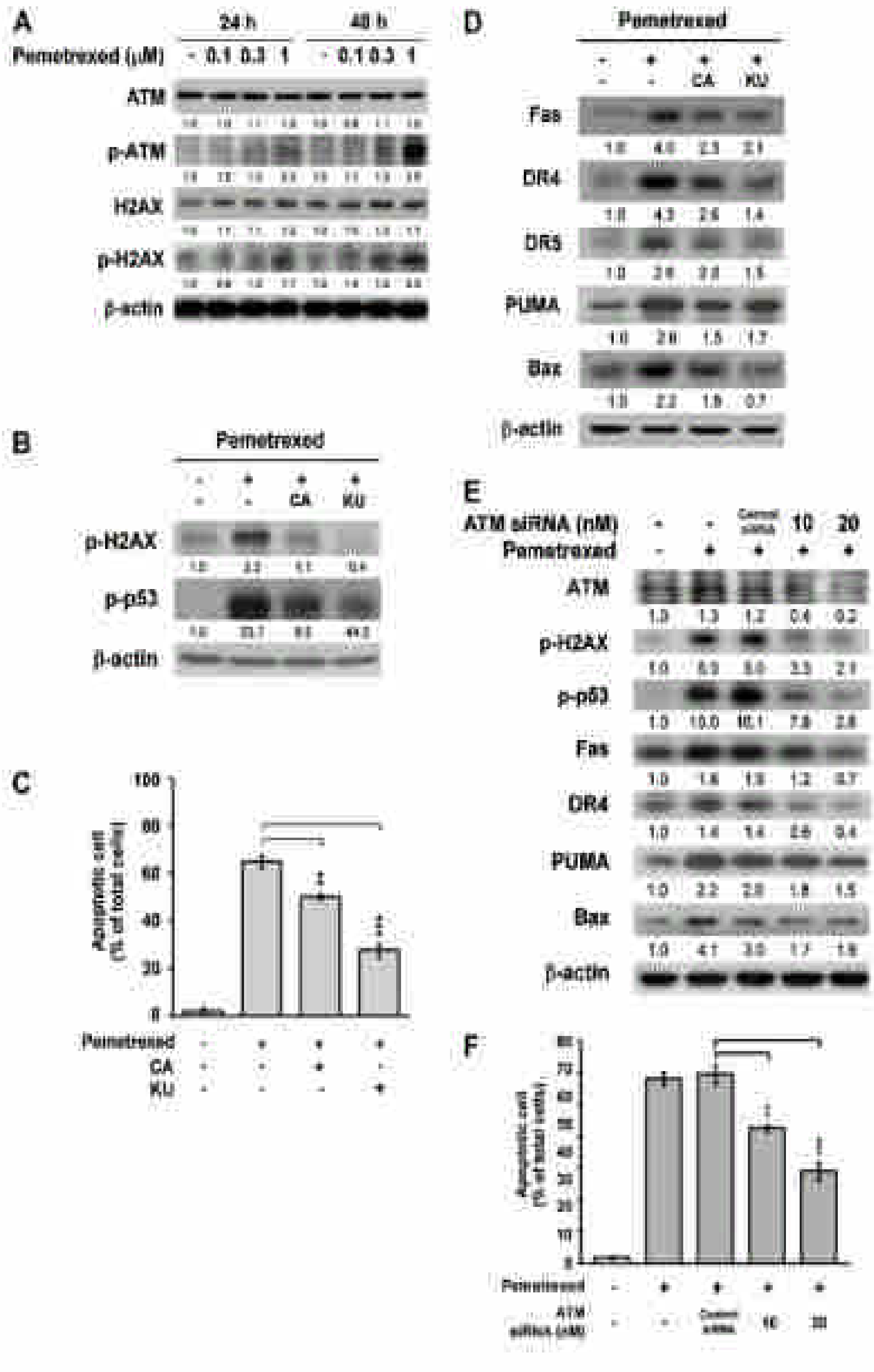

Figure 5. ATM acts as an upstream regulator in response to pemetrexed-induced apoptosis. (A) Pemetrexed induced the phosphorylation of ATM and H2AX. The expression of ATM, phospho-ATM, H2AX, and phospho-H2AX in A549 cells was detected at 24 and 48 h with indicated concentrations of pemetrexed. An equal amount of cell lysate was analyzed by Western blotting with β-actin as the loading control. Pharmacological inhibition of ATM and ATM knockdown suppressed the phosphorylation of H2AX and p53 (B and E), blocked the induction of apoptosis (C and F), and decreased the expression of proapoptotic molecules (D and E) in response to pemetrexed treatment. A549 cells were pretreated with or without caffeine (CA, 1 mM), KU55933 (KU, 3 μM) or ATM siRNA (10 or 20 μM) for various times and were then further treated with 1 μM of pemetrexed for another 48 h. (E) Western blot analysis was performed as described above. Apoptotic cell death was determined by TUNEL assay. Results are represented as means ± SD for three independent experiments. $^{**}P < 0.01$, $^{**}P < 0.05$, $^{***}P < 0.001$.

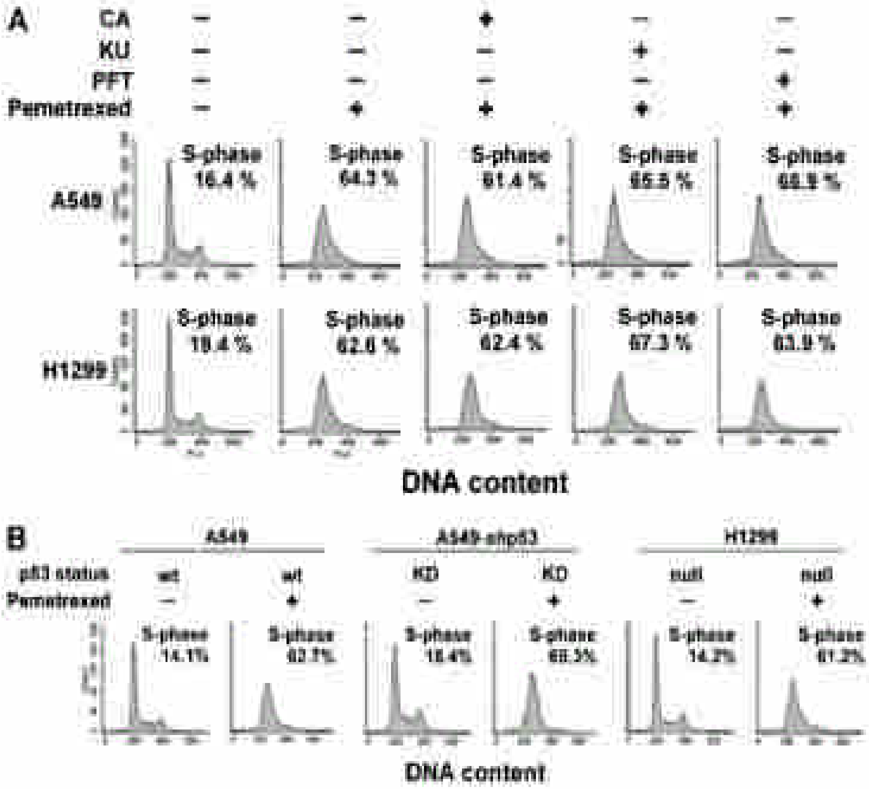

Figure 6. Inhibition of ATM and p53 has no effect on pemetrexed-induced S-phase arrest. (A) A549 cells were pretreated with or without CA (1 mM) or KU (3 μM) for 20 min, and then 1 μM of pemetrexed was added. The cell cycle distribution was analyzed by flow cytometry after 24 h of pemetrexed treatment. (B) A549, A549-shp53, and H1299 cells were treated with or without pemetrexed (1 μM) for 24 h, and the cell cycle distribution was analyzed by flow cytometry.

identify the surrogate biomarkers for evaluating the treatment efficacy of pemetrexed in vitro. Our previous study demonstrated that the antitumor activities of pemetrexed are mediated by the activation of ERK and Cdk2/cyclin-A pathways to induce S-phase arrest and apoptosis in human non-small cell lung cancer A549 cells [23]. In the present study, we further characterized the mechanistic action of pemetrexed in A549 cells. We found that dTTP depletion-mediated S-phase arrest, DNA damage, and activation of phosphorylated ATM, γH2AX, and p53 were early consequences of pemetrexed exposure. Pemetrexed treatment upregulated death receptor family molecules (Fas,

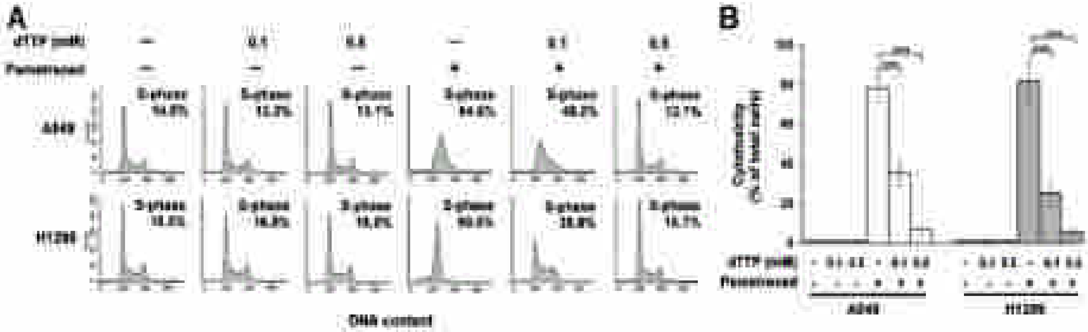

Figure 7. Pemetrexed-mediated cell cycle arrest and death were reversed by dTTP. (A) A549 or H1299 cells were pretreated with the indicated concentrations of dTTP for 12 h, and then 1 μM pemetrexed was added. The cell cycle distribution was analyzed by flow cytometry after 24 h of pemetrexed treatment. (B) Cells were pretreated with dTTP and then treated with 1 μM pemetrexed for 72 h, cell viability was measured by the Trypan blue dye exclusion method. Results are mean ± SD for three independent experiments. $^{***}P < 0.001$

Fas ligand, DR4, and DR5) and Bcl-2 family proteins (PUMA and Bax) and subsequently activated the caspase cascade, leading to both intrinsic and extrinsic apoptotic cell death. Some of these results have been confirmed in H1299 cells, suggesting that pemetrexed may activate some common signaling events in lung cancer cells.

The DNA damage response is a highly orchestrated and complex signaling event [24]. Many DNA-damaging agents, especially those that generate DNA double-strand breaks, are known to activate ATM and induce ATM-dependent apoptosis [25]. In response to DNA damage, ATM rapidly phosphorylates its downstream targets, including histone H2AX and p53 at Ser139 and Ser15, respectively [20,21,26,27]. Pemetrexed induces ATM autophosphorylation in human mesothelioma cell lines, and CA-mediated sensitization of pemetrexed activity in these cells is associated with an increase in pemetrexed-induced ATM phosphorylation [28]. The present study showed that pemetrexed induced cell cycle S-phase arrest, DNA damage (Figures 1A and 6), and subsequent phosphorylation of ATM^{S1981}, $\gamma H2AX^{S139}$, and $p53^{S15}$ (Figure 5A). Functionally, we found that ATM signaling was largely responsible for $\gamma H2AX^{S139}$ and $p53^{S15}$ phosphorylation and activation during pemetrexed treatment. As a result, inhibition of ATM by pharmacological inhibitors or genetic knockdown suppressed $\gamma H2AX^{S139}$ and $p53^{S15}$ phosphorylation under our experimental conditions (Figure 5B and E). Our results further support the significance of the ATM pathway in pemetrexed-mediated injurious events, including PUMA and Fas induction, and apoptosis, which were reduced following ATM inhibition (Figure 5C–F). However, inhibition and knockdown of ATM only partially blocked pemetrexed-mediated cell death. Therefore, the possibility that the pemetrexed-induced cytotoxicity may also involve other molecules cannot be excluded.

The molecule p53 plays a critical role in the cellular response to DNA damage by regulating genes involved in cell cycle progression, genomic instability, and apoptosis [29]. The importance of p53 in chemotherapy-induced apoptosis of cancer cells is well established. It is noted that greater than 50% of lung tumors lose p53 function [30–32]. Previous studies demonstrated that treatment with antifolate drugs induce DNA damage and p53 activation [12,33,34]. Moreover, the status of p53 is an important determinant of pemetrexed sensitivity in colon cancer cell lines [35] and in patients with colorectal cancer [36]. Conversely, several studies have suggested that pemetrexed-induced cell death is independent of the presence of wild-type p53 [37,38]. In the current study, A549 cells that were treated with p53 inhibitor PFT became less (not more) sensitive to pemetrexed-induced cytotoxicity (Figure 4B). The possible impact of p53 status on the sensitivity to pemetrexed was further investigated using one pair of related cell lines (A549 and A549/p53-shRNA cells) and p53-null H1299 cells, which revealed that functional p53 status was not associated with cellular toxicity response to pemetrexed (Figures 4C and 6B). Based on our data, we conclude that p53 status alone is not a useful predictor of pemetrexed efficacy.

The activation of caspase cascade is one of the biomarkers of apoptosis [39]. Our data clearly demonstrated that pemetrexed-associated apoptosis is mediated through the caspase activation pathway in A549 cells. Two distinct pathways upstream of the caspase cascade have been identified: (1) death receptor-induced apoptosis (extrinsic pathway), and (2) mitochondrial stress-induced apoptosis (intrinsic pathway) [40]. Death receptors (e.g., Fas, TNF-R, and TRAIL-R: DR4, DR5) trigger caspase-8 activation, and the Bcl-2 family molecule-provoked mitochondria events activate caspase-9. In human colorectal cancer cell lines, pemetrexed-induced apoptosis is accompanied by increased Bax protein expression and caspase-9/-3 activation [34]. In multiple myeloma cell lines, pemetrexed-triggered cell death is associated with the regulation of Bcl-2 family proteins and the activation of caspase-8, -9, and -3 [41]. Our study in human non-small cell lung cancer A549 cells revealed that pemetrexed induced Bax and PUMA upregulation. Silencing Bax expression by shRNA or ectopic expression of Bcl-2 in A549 cells effectively inhibited pemetrexed-mediated apoptosis. These suggest that Bcl-2 family molecules are required for pemetrexed-mediated cytotoxicity. In addition, our current study showed that Fas and TRAIL receptors (DR4 and DR5) were increased after pemetrexed treatment. The Fas neutralizing antibody and DR4:Fc antagonist obviously inhibited pemetrexed-induced cell death, indicating that the death receptor-mediated extrinsic apoptotic pathway was also a necessary process in pemetrexed-triggered cell death. These findings agree with those of Longley et al. [33], who have shown that the Fas signaling pathway is an important mediator of pemetrexed-induced cell death. Moreover, we demonstrated that upregulated PUMA and death receptors (Fas, DR4, and DR5) were accompanied by pemetrexed-mediated cytotoxicity in H1299 cells. As previously shown, transcription of PUMA and death receptor genes may be regulated by p53-dependent as well as -independent mechanisms [42]. Our results indicate that pemetrexed induced PUMA and death receptor expression in H1299 cells, which lack functional p53, suggesting a p53-independent mechanism.

A variety of DNA-damaging agents, including γ-irradiation and doxorubicin, have been shown to induce either G1 or G2 arrest, which is mediated by p53 [43–45]. The current study showed that pemetrexed induced DNA damage with S-phase cell cycle arrest and ATM/p53 activation. Therefore, it was important to assess the role of the ATM/p53 activation in pemetrexed-mediated S-phase arrest. However, inhibition of ATM and p53 by a pharmacological inhibitor and siRNA-based knockdown did not affect the pemetrexed-induced S-phase arrest in A549 cells (Figure 6), suggesting that ATM and p53 played a passive role in the pemetrexed-induced perturbations in the cell cycle. Furthermore, pemetrexed treatment in a p53-null lung cancer cell line, H1299 cells, also caused S-phase arrest. Accordingly, pemetrexed-induced S-phase arrest appeared to be a universal event regardless of the p53 phenotype. We also demonstrated that the phosphorylation of ATM and p53 in response to pemetrexed-treatment was delayed and could only be detected after exposure times of at least 24–48 h (Figures 4A and 5A). Therefore, the early detection of S-phase arrest and comet formation (Figures 1A and 6A) in the absence of ATM, H2AX, and p53 phosphorylation in pemetrexed-exposed cells is consistent with a mechanism of pemetrexed-mediated genotoxicity that does not result in direct DNA strand breaks, but involves the depletion of dTTP pools leading to the S-phase arrest, which perturbs the progression of DNA-replication forks and causes DNA double-strain damage. This was confirmed by demonstrating that, when supplemented with exogenous dTTP, the pemetrexed-treated cells were able to immediately resume a normal DNA replication (Figure 7A). Similar results have been previously reported and show that pemetrexed-dependent DNA damage originates from dTTP depletion followed by its reduced incorporation into DNA and subsequent S-phase arrest [46].

Based on our findings, a signaling cascade is proposed in Figure 8. Pemetrexed-induced inhibition of TS in non-small cell lung cancer causes a massive dTTP depletion followed by S-phase arrest and genotoxic stress. In addition, pemetrexed activates DNA damage/stress response genes, including ATM, γH2AX^{ser139}, and p53^{ser15}. Pemetrexed induces the expression of PUMA, which facilitates the mitochondrial translocation of Bax, leading to caspase-9/-3 activation and apoptosis. Meanwhile, pemetrexed increases the expression of Fas and TRAIL receptors followed by caspase-8/-3 activation and apoptosis. Importantly, inhibition of ATM and p53 partially suppresses the expression of PUMA and death receptors and attenuates pemetrexed-induced cytotoxicity, suggesting that both ATM/p53-dependent and -independent pathways contributed to drug activity. Because some of the results have been confirmed in H1299 cells, we hypothesized that pemetrexed may activate some

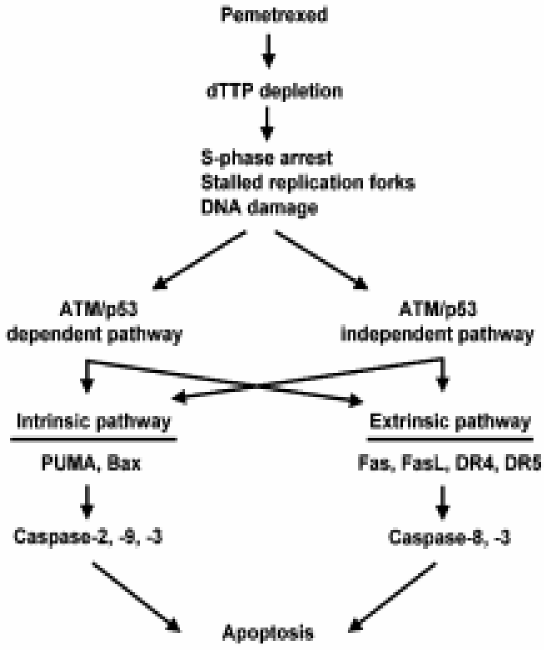

Figure 8. Schematic diagram of the molecular events of pemetrexed-mediated apoptosis in human lung adenocarcinoma A549 cells. The depletion of dTTP levels by pemetrexed would cause S-phase cell cycle arrest and DNA damage followed by the activation of ATM/p53-dependent and -independent signaling pathways, which promoted intrinsic and extrinsic apoptotic cell death by upregulating Bax, PUMA, Fas, DR4, and DR5 and activating the caspase signaling cascade.

common signaling events in different non-small cell lung cancer cell types.

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國科會補助計畫衍生研發成果推廣資料表

日期:2012/10/02

<table>
<tr><td rowspan="3">國科會補助計畫</td><td>計畫名稱: 反轉肺腺癌細胞對多重抗葉酸類抗癌藥Pemetrexed(愛寧達)之抗藥性的可行性</td></tr>
<tr><td>計畫主持人: 許國堂</td></tr>
<tr><td>計畫編號: 100-2320-B-040-005-　　　　學門領域: 藥理及毒理</td></tr>
<tr><td colspan="2">無研發成果推廣資料</td></tr>
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100 年度專題研究計畫研究成果彙整表

計畫主持人：許國堂	計畫編號：100-2320-B-040-005-
計畫名稱：反轉肺腺癌細胞對多重抗葉酸類抗癌藥 Pemetrexed(愛寧達)之抗藥性的可行性	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	2	2	100%	人次	
		博士生	2	2	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	1	2	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
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	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：（以 100 字為限）

已經發表壹篇期刊論文(Pemetrexed induces both intrinsic and extrinsic apoptosis through ataxia telangiectasia mutated/p53-dependent and -independent signaling pathways.) Yang TY, Chang GC, Chen KC, Hung HW, Hsu KH, Wu CH, Sheu GT, Hsu SL. Mol Carcinog. 2011 Nov 15. [Epub ahead of print]. IF: 3.265 (ONCOLOGY 63/184, 34.2%). 尚有部份數據正在補充，希望可以再發發表壹篇期刊論文。

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

Pemetrexed (愛寧達，Alimta)為葉酸拮抗劑抗癌藥物，其引起癌細胞凋亡作用機轉可以經由 intrinsic and extrinsic apoptosis by upregulating Bax, PUMA, Fas, DR4, and DR5 and activating the caspase signaling cascade.所以癌細胞對愛寧達的敏感性可以檢驗 thymidylate synthase 的降低和 Bax, PUMA, Fas, DR4, and DR5 之增加來預測而得到較好的治療效果。自 2009 年二月起，衛生署已經核准愛寧達可用於肺腺癌的第一線化療用藥，讓肺癌病患在化療第一時間就可以選用愛寧達，不必像過去，得等到第一線化療失敗後才能使用。可是目前對於愛寧達之抗藥性產生之機轉並無深入的研究，因此，研究肺癌對愛寧達之抗藥性產生之機轉就顯得非常需要。