EUROPEAN JOURNAL OF CANCER 47 (2011) 2471–2478

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Death Receptor 5 and cellular FLICE-inhibitory protein regulate pemetrexed-induced apoptosis in human lung cancer cells

Ling Su [a,f], *Guangbo Liu* [a,f], *Xuexi Hao* [b,f], *Ning Zhong* [c], *Diansheng Zhong* [d], *Xiangguo Liu* [a,*], *Sunil Singhal* [e]

[a] *School of Life Sciences, Shandong Provincial Key Laboratory Of Animal Cells and Developmental Biology, Shandong University, Jinan, People's Republic of China*
[b] *The Second Hospital, Shandong University, Jinan, People's Republic of China*
[c] *School of Medicine, Shandong University, Jinan, People's Republic of China*
[d] *Tianjin Key Laboratory of Lung Cancer Metastasis and Tumor Environment, Tianjin Lung Cancer Institute, Tianjin Medical University General Hospital, Tianjin, People's Republic of China*
[e] *Division of Thoracic Surgery, Department of Surgery, University of Pennsylvania, Philadelphia, PA, USA*

ARTICLE INFO

Article history:
Available online 2 July 2011

Keywords:
Pemetrexed
DR5
c-FLIP
Apoptosis
CHOP
TRAIL

ABSTRACT

Pemetrexed is a clinically available anti-folate therapeutic agent used in combination with cisplatin for the management of patients with malignant pleural mesothelioma and advanced non-small cell lung cancer. Pemetrexed inhibits three enzymes in purine and pyrimidine synthesis necessary for precursor DNA nucleotides which in turn disrupts growth and survival of normal and cancer cells. The mechanism by which pemetrexed induces apoptosis remains largely uncharacterised. In the current study, we examined the downstream effect of pemetrexed in inducing apoptosis in lung cancer cells. We showed that pemetrexed induced apoptosis via up-regulation of Death Receptor 5 (DR5), an important death receptor for tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL). In addition, we discovered a synergistic effect of combination pemetrexed and recombinant TRAIL in inducing apoptosis. Modulating DR5 induction by small interfering RNA abrogated the ability of pemetrexed to induce apoptosis. In addition, silencing of C/EBP homologous protein (CHOP) expression reduced DR5 expression, demonstrating that the transcriptional factor CHOP has a pivotal role on DR5 up-regulation following pemetrexed treatment. In addition, enforced expression of cellular FLICE-inhibitory protein (c-FLIP), a known inhibitor of caspase 8, protected neoplastic cells from apoptosis despite pemetrexed and/or TRAIL therapy. Thus, our findings demonstrate the efficacy and mechanistic underpinnings of pemetrexed-induced apoptosis, and they suggest pemetrexed may have clinical utility when used in combination with TRAIL for the management of patients with lung cancer.

1. Introduction

Non-small cell lung cancer (NSCLC) is the most common cancer in the United States (>200,000 cases/year), and carries a dismal 5 years 15% survival.[1] Surgery is available for a limited number of patients, and chemotherapy remains the mainstay of therapy for this disease. Cisplatin-based regimens are the standard of care in combination with various other

* *Corresponding author:* Address: Shandong University, School of Life Sciences, Room 103, South Building, 27 Shandananlu Road, Jinan 250100, People's Republic of China. Tel.: +86 531 88364330.
E-mail address: xgliu@sdu.edu.cn (X. Liu).
[f] Ling Su, Guangbo Liu, Xuexi Hao contributed equally to this work, thus share the first authorship.
0959-8049/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2011.06.003

agents such as pemetrexed. Pemetrexed (Alimta®) is a multi-targeted anti-folate cytotoxic agent available for use in the treatment of patients with NSCLC. It is indicated as initial therapy in combination with cisplatin, as monotherapy after prior chemotherapy, and as maintenance monotherapy in patients with locally advanced or metastatic non-squamous NSCLC.[2]

Pemetrexed causes cell cycle arrest[3–6] and apoptosis.[7–9] Pemetrexed blocks three enzymes used in purine and pyrimidine synthesis—thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT). As a consequence, inhibition of the cellular DNA machinery via disruption of folic acid metabolism prevents cellular division and replication.[3–6] However, to date, the mechanistic underpinnings of directed cancer cell apoptosis by pemetrexed have not been fully elucidated. Previous studies have suggested that pemetrexed-induced apoptosis is associated with p53 and inactivation of bcl-2.[7–9] However, targeted cancer cell apoptosis for this widely used clinical agent remains largely unexplained.

There are two major apoptosis signalling pathways: the intrinsic mitochondria-mediated pathway and the extrinsic death receptor–induced pathway.[10] The truncated form of the pro-apoptotic protein Bid serves as the cross-talk between these two pathways. Death Receptor 5 (DR5) is an important mediator of the extrinsic apoptotic signalling pathway. When present locally in the tumour microenvironment, tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) binds DR5 and preferentially induces apoptosis in transformed cells while sparing normal cells, in contradistinction to other mediators of programmed cell death such as TNF and FasL.[11,12] DR5 expression is inducible by cancer therapeutic and preventive agents, and up-regulation of DR5 often explains induction of programmed cell death or augmentation of TRAIL-induced apoptosis.[13–15]

A central step in the execution of apoptosis is the activation of caspases, which are widely present as inactive forms.[10] Cellular FLICE-inhibitory protein (c-FLIP) is the pivotal protein that negatively modulates the caspase cascade. Specifically, caspase 8 is activated by death receptors through Fas associated death domain (FADD) binding of the death inducing signalling complex (DISC). Thus, the primary role of c-FLIP is a specific inhibitor of death receptor-mediated apoptosis.[16] Accordingly, down-regulation of c-FLIP confers sensitivity to death receptor-induced apoptosis. Although multiple splicing isoforms of c-FLIP mRNA have been reported, c-FLIP$_L$ and c-FLIP$_S$, are major splicing variants detectable at the protein level and have been extensively characterised.[17]

In this study, we explore the mechanism of pemetrexed-induced apoptosis and the role of Death Receptor 5 and cellular FLICE-inhibitory protein as regulators of the programmed cell death of cancer cells.

2. Materials and methods

2.1. Reagents

Pemetrexed was purchased from Toronto Research Chemicals, Inc. (Ontario, Canada). Caspase 3 antibody was obtained from Imgenex (San Diego, CA). TRAIL was purchased from Merck (Whitehouse Station, NJ). Caspase 8, caspase 9, Bip and IRE1α antibodies were purchased from Cell Signaling Technology (Danvers, MA). C/EBP homologous protein (CHOP) antibody was obtained from Santa Cruz (Santa Cruz, CA). DR5 antibody was obtained from ProSci (Poway, CA).

2.2. Cell lines and cell culture

All human lung cancer cell lines throughout this study were purchased from the American Type Culture Collection (Manassas, VA). These cell lines were cultured in RPMI 1640 medium containing 5% foetal bovine serum at 37 °C in a humidified atmosphere of 5% CO2 and 95% air.

2.3. Western blot analysis

Preparation of whole-cell protein lysates and the procedures for the Western blotting were previously described.[13]

2.4. Silencing of DR5 and CHOP expression with siRNA

siRNAs were synthesised by GenePharma (Shanghai, China). DR5 and CHOP siRNA target sequences were described before.[13,18] The transfection of siRNA was conducted as previously described.[13]

2.5. Establishment of stable cell lines that overexpress c-FLIP$_L$

c-FLIP$_L$ coding regions were amplified by PCR and cloned into the pLenti6 vector (Invitrogen) following the manufacturer's protocol. The lentivirus was prepared in 293FT cells and A549 cell clones were selected as previously described.[19] For brevity, the clone that can overexpress c-FLIP$_L$ is referred to as A549-FLIP$_L$, and the control clone is labelled A549-LacZ.

2.6. Apoptosis assays

Apoptosis was evaluated by Annexin V staining using Annexin V-PE/7-AAD apoptosis detection kit purchased from BD Biosciences (San Jose, CA) following the manufacturer's instructions. Caspase activation was detected by Western blotting.

3. Results

3.1. Pemetrexed up-regulates DR5 expression in human NSCLC cells

To characterise the mechanism through which pemetrexed induces apoptosis, three NSCLC cell lines (H157, A549, H1792) were treated with pemetrexed and examined for cellular apoptosis using Annexin V/7-AAD staining and by flow cytometry analysis. The apoptosis of all three cell lines was effectively induced by 48 h of pemetrexed treatment (2.5 μmol/L) (Fig. 1), suggesting induction of apoptosis may account for growth inhibition of human lung cancer cells.

To determine the molecular signalling pathway for pemetrexed-induced apoptosis, we screened the expression of apoptotic related genes in cells treated with pemetrexed. We found pemetrexed could cleave caspase 8, caspase 9,

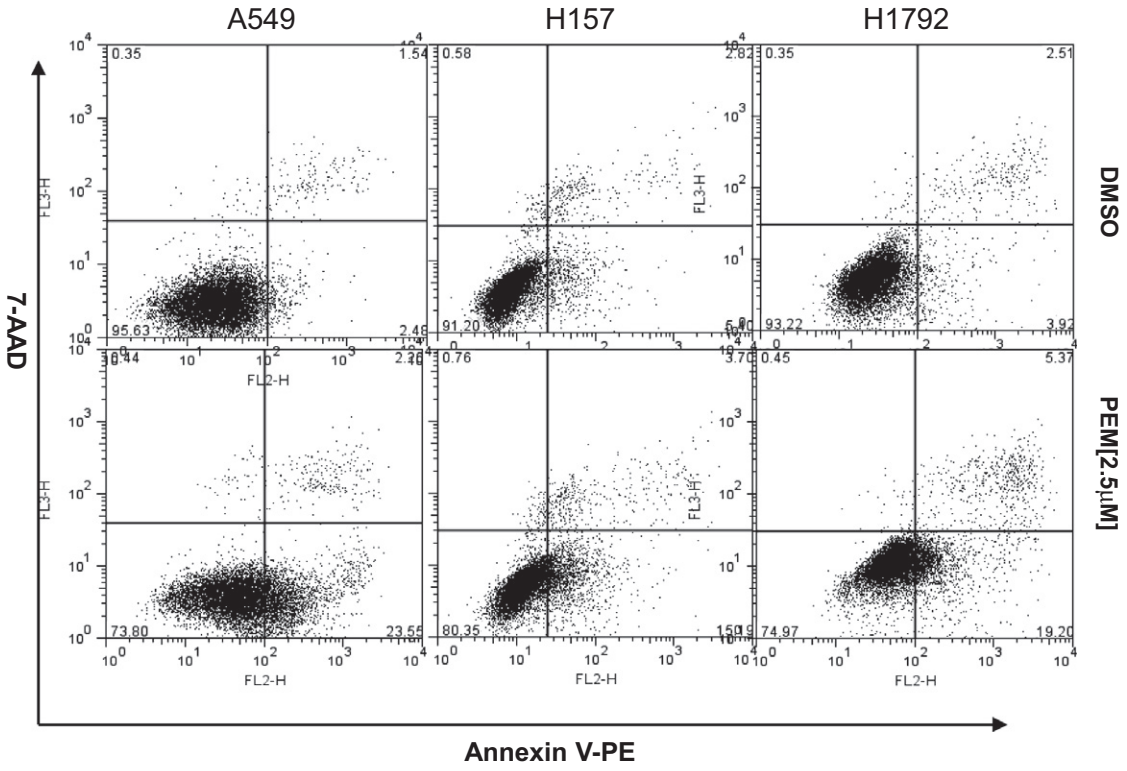

Fig. 1 – Pemetrexed induces apoptosis in non-small cell lung cancer (NSCLC) cells. The indicated cell lines were treated with and without 2.5 μmol/L pemetrexed (PEM) for 48 h. Cells were subjected to Annexin V-PE/7-AAD staining and flow cytometry analysis.

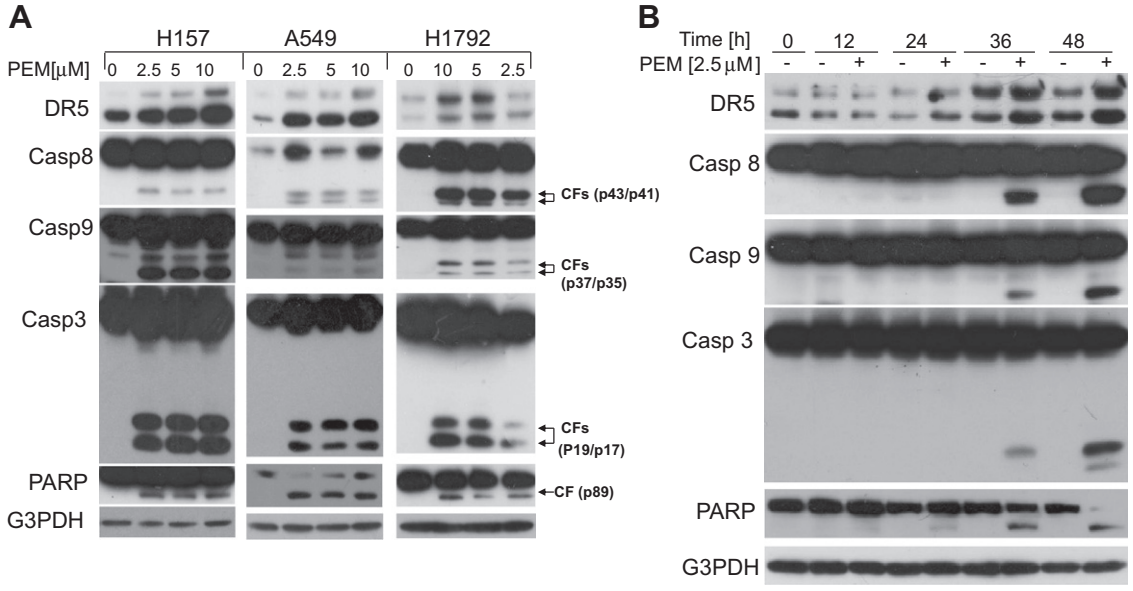

Fig. 2 – Pemetrexed activates caspases and induces DR5 expression in NSCLC cells in a dose-dependent (A) and a time-dependent manner (B). The indicated cell lines were treated with the given concentrations for 48 h. The cells were then subjected to a preparation of whole-cell protein lysates and subsequent Western blot analysis (A). Time-dependent effects of PEM on the expression of DR5, caspase 8, caspase 9, caspase 3 and PARP in A549 cells (B). The cells were treated with 2.5 μmol/L for various times from 12 to 48 h as indicated and then subjected to the preparation of whole-cell protein lysates and subsequent Western blot analysis for the given proteins (B).

caspase 3 and the substrate of caspase 3, poly (ADP-ribose) polymerase (PARP). Moreover, DR5 was also discovered to be up-regulated in cells exposed to pemetrexed. With increasing pemetrexed dosing, we found DR5 protein levels increased in a dose-dependent manner (concentrations ranging from 2.5 to 10 μmol/L) in H157, A549 and H1792 (Fig. 2A). Time course analysis indicated that pemetrexed increased DR5 levels at 36 h (h) and reached the peak protein expression at 48 h following pemetrexed treatment in A549 cells (Fig. 2B).

To determine if DR5 inhibition conferred NSCLC resistance to pemetrexed-induced apoptosis, DR5 gene expression was silenced using RNAi methodology. DR5 siRNA transfection decreased both the basal level of DR5 expression and the level of pemetrexed-increased DR5 expression and decreased the pemetrexed-increased levels of cleaved caspase 8, 9, 3 and PARP (Fig. 3A). The percent of apoptotic A549 cells following pemetrexed treatment decreased from 29% to 18% relative to A549 cells treated with DMSO (Fig. 3B). In summary, our findings suggest pemetrexed treatment up-regulates DR5 and can result in apoptosis in lung cancer cells.

3.2. *CHOP gene silencing down-regulates DR5 expression and reduces pemetrexed-induced apoptosis*

The induction of DR5 expression generally occurs at the transcriptional level, including activation of the transcriptional factors such as CHOP (C/EBP homologous protein; also known as growth arrest and DNA damage gene 153, GADD153).[20] We hypothesised CHOP may also be important for DR5 induction after pemetrexed treatment. We found that CHOP levels increased at 48 h following pemetrexed treatment in a dose-dependent manner (Fig. 4A). Time course analysis demonstrated that CHOP increased 24 h following pemetrexed treatment and remained high for 48 h (Fig. 4B). CHOP siRNA transfection decreased the induction level of CHOP expression and suppressed the elevated levels of cleaved caspase 8, caspase 9, caspase 3 and PARP despite pemetrexed treatment (Fig. 4C). The percent of apoptotic cells declined from 32% to 22%, suggesting CHOP plays a critical role in the upregulation of DR5 after pemetrexed exposure (Fig. 4D).

Since CHOP plays an important role in apoptosis mediated by endoplasmic reticulum (ER) stress, we postulate that pemetrexed induces ER stress in lung cancer cells. Bip and IRE1α are generally regarded as ER stress markers.[20] We treated A549 and H157 cells with pemetrexed for 48 h and quantitated Bip and IRE1α levels using Western Blot technology. We found Bip and IRE1α expression levels to be increased in a concentration-dependent manner (Supplementary Fig. 1S), suggesting pemetrexed treatment results in ER stress in lung cancer cells. These data hint that pemetrexed up-regulates CHOP through ER stress induction.

3.3. *Enforced ectopic c-FLIP$_L$ expression protects NSCLC cells from apoptosis*

Since FLIP$_L$ is a key protein that negatively regulates the extrinsic death receptor-mediated apoptotic pathway by inhibiting caspase-8 activation, some cancer therapeutic agents enhance cell sensitivity to TRAIL-induced apoptosis via down-regulation of c-FLIP expression.[16,17,19] Therefore,

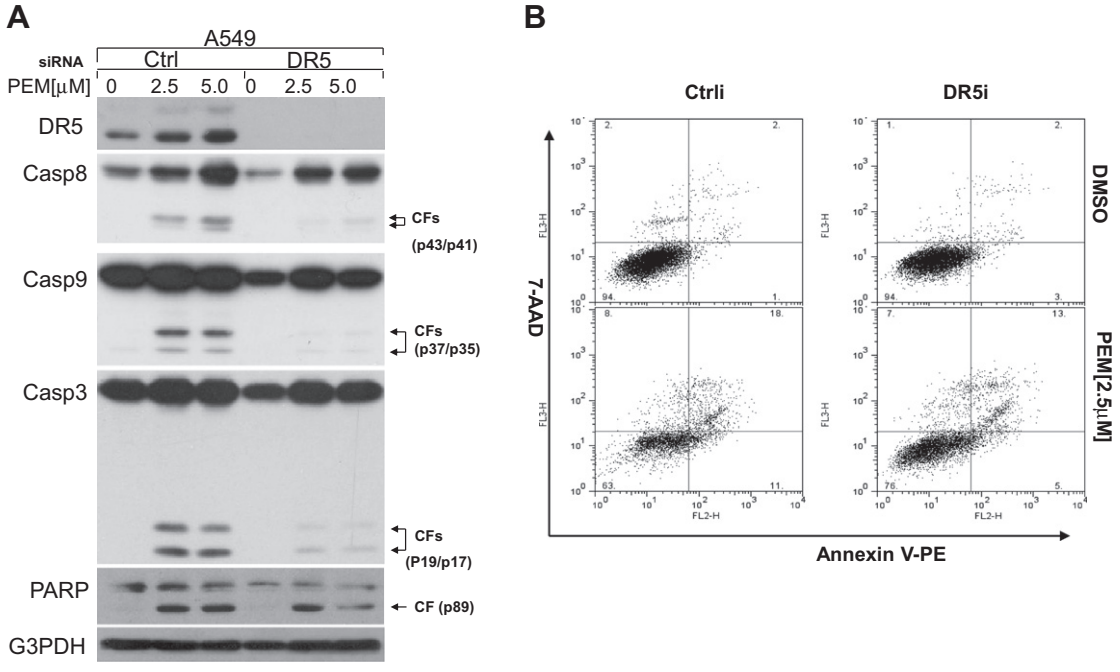

Fig. 3 – Silencing of DR5 expression by siRNA (A) attenuates apoptosis induced by pemetrexed (B). A549 cells were seeded in a 6-well cell culture plate and on the second day transfected with control (Ctrl) or DR5 siRNA. Forty-eight hours later, the cells were treated with 2.5 μmol/L pemetrexed. After 48 h, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis (A) or for detection of apoptotic cells using annexin V/7-AAD staining (B). In annexin V/7-AAD assay, the percent positive cells in the top right and bottom right quadrants were added to yield the total of apoptotic cells. The cells in the bottom left quadrant were viable cells.

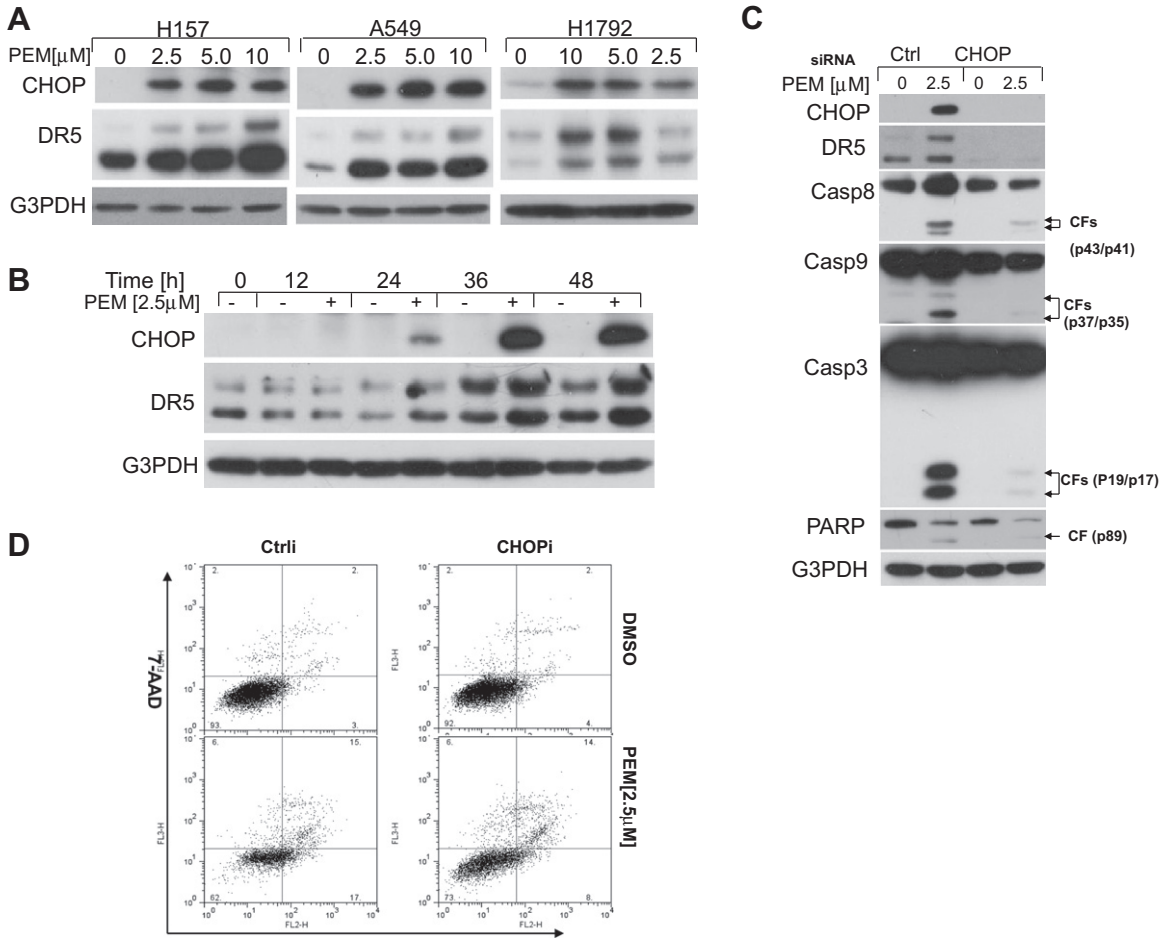

Fig. 4 – Pemetrexed induces CHOP and DR5 up-regulation in a dose-dependent (A) and a time-dependent manner (B), and silencing of CHOP expression by siRNA (C) attenuates apoptosis induced by pemetrexed (D). The indicated cell lines were treated with the given concentrations for 48 h (A). A549 cells were treated with 2.5 μmol/L for various times from 12 to 48 h as indicated (B). The cells in A and B were subjected to the preparation of whole-cell protein lysates and subsequent Western blot analysis for the CHOP and DR5 expression. A549 cells were seeded in a 6-well cell culture plate and on the second day transfected with control (Ctrl) or CHOP siRNA. Forty-eight hours later, the cells were treated with 2.5 μmol/L pemetrexed. After 48 h, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis (C) or for detection of apoptotic cells using Annexin V/7-AAD staining (D).

we further examined the effects of pemetrexed on c-FLIP expression in human lung cancer cells. We found that pemetrexed treatment could down-regulate c-FLIP$_L$ in a concentration-dependent manner in the cell lines H157, A549 and H1792 which we detected (Fig. 5A). In order to determine if c-FLIP$_L$ is important to pemetrexed-mediated apoptosis, we used a lentiviral expression system to enforce expression of c-FLIP$_L$ in A549 cell line and then analysed its effect on apoptosis induced by pemetrexed. We found that the levels of cleaved caspase 8, caspase 9, caspase 3 and PARP in A549-FLIP$_L$ were reduced compared with A549-LacZ control cells (Fig. 5B). This demonstrated that c-FLIP$_L$ could prevent the cells from apoptosis following pemetrexed therapy.

3.4. *Enforced expression of ectopic c-FLIP$_L$ protects NSCLC cells from induction of apoptosis when combined with TRAIL*

It has been reported that many chemotherapeutic agents in combination with TRAIL augment the induction of apoptosis in human non-small cell lung cancer cells.[21,22] To determine whether down-regulation of c-FLIP also contributes to synergy between pemetrexed and TRAIL on apoptosis induction, we examined the effects of enforced overexpression of c-FLIP$_L$ on pemetrexed-mediated enhancement of TRAIL-induced apoptosis in A549 lung cancer cell lines. In order to detect the synergy between these two agents, we used pemetrexed (2.5 μmol/L) and TRAIL (40 ng/ml) to treat A549-LacZ or A549-FLIP$_L$ cells. Cells were treated for 24 h in order for adequate synergy between pemetrexed and TRAIL. Within 24 h, we could detect high levels of ectopic c-FLIP$_L$ (Fig. 6A). In contradistinction, pemetrexed alone typically induces apoptosis after 36 h[7] suggesting this approach does not cleave any pro-apoptotic proteins by 24 h. However, the combination of pemetrexed and TRAIL induced cleavage of caspase 8, caspase 9, caspase 3 and PARP in A549-lacZ control cells. This was confirmed by the decline in proform levels and the increase in their cleaved bands. Importantly, these effects were diminished in A549-FLIP$_L$ cells. In repeat experiments, we

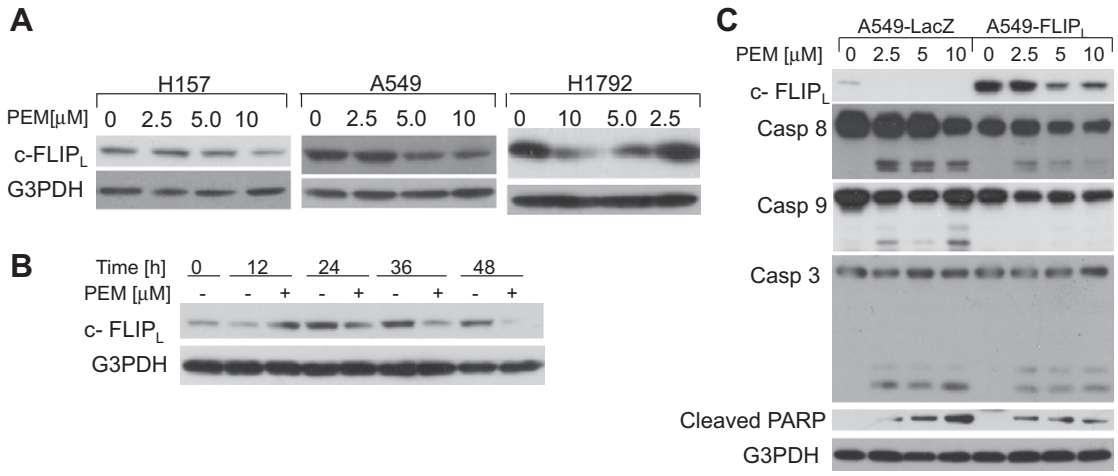

Fig. 5 – Pemetrexed degrades c-FLIP$_L$ in NSCLC cells in a dose-dependent (A) and a time-dependent manner (B), and enforced c-FLIP expression protects the cells from apoptosis induced by PEM treatment (C). The indicated cell lines were treated with the given concentrations for 48 h (A). The cells were then subjected to the preparation of whole-cell protein lysates and subsequent Western blot analysis for the c-FLIP$_L$ expression. Time-dependent effects of PEM on the c-FLIP$_L$ expression (B). The cells were treated with 2.5 μmol/L for various times from 12 to 48 h as indicated and then subjected to the preparation of whole-cell protein lysates and subsequent Western blot analysis for c-FLIP$_L$ expression (B). A549-LacZ and A549-FLIP$_L$ cells were treated with DMSO, 2.5, 5, 10 μmol/L PEM, respectively. After 48 h, c-FLIP$_L$ expression and caspase activation were measured by Western blot analysis (C).

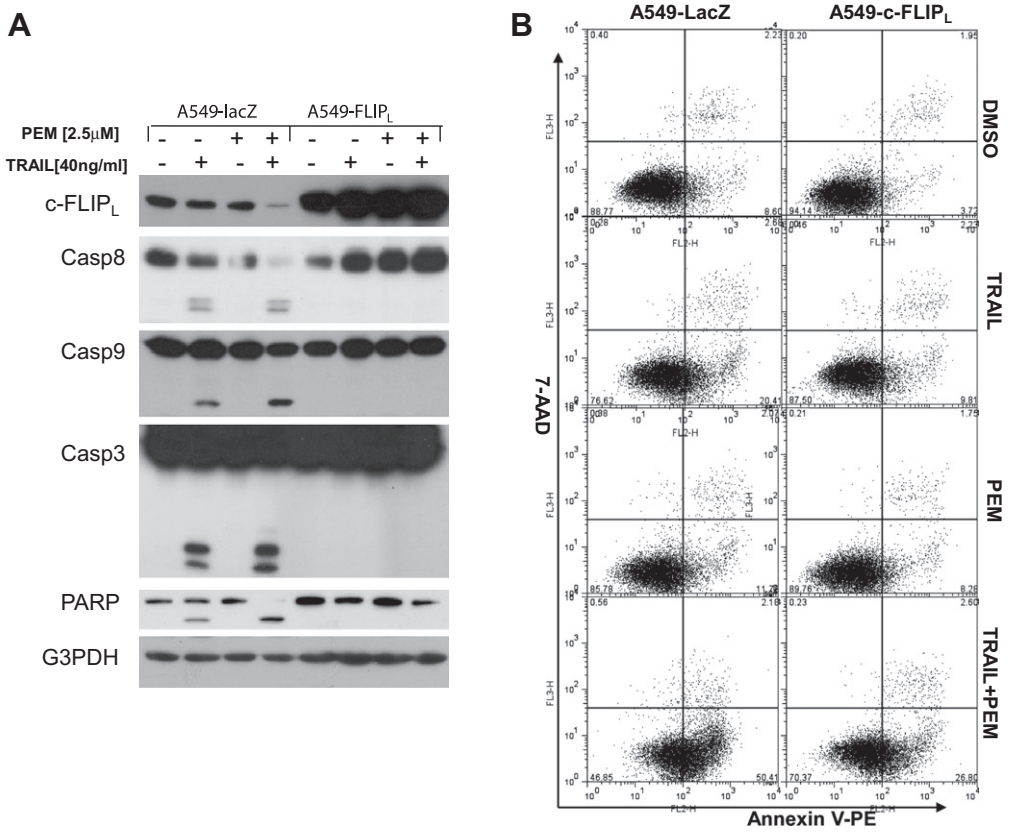

Fig. 6 – Enforced c-FLIP$_L$ expression protects the cells from apoptosis induced by the combination of PEM and TRAIL (B). A549-LacZ and A549-FLIPL cells were treated with 2.5 μmol/L PEM, 40 ng/mL TRAIL, and their combination. After 24 h, the cells were harvested for the preparation of whole-cell protein lysates and Western blot analysis (A) and detection of apoptotic cells using Annexin V staining and flow cytometry analysis (B).

measured 53.2% apoptosis in A549-LacZ cells treated with combination of TRAIL and pemetrexed, but only 29.4% apoptosis in A549-FLIP$_L$ cells after the same treatment (Fig. 6B). Thus, these results collectively show that down-regulation of c-FLIP$_L$ contributes to pemetrexed-mediated enhancement of TRAIL-induced apoptosis.

4. Discussion

Non-small cell lung cancer (NSCLC) is a devastating disease with dismal prognosis that is predominately treated by chemotherapy when patients are not surgical candidates. Pemetrexed is commonly used to manage this disease. In this study, we demonstrate that pemetrexed induces apoptosis in human NSCLC cells and sensitises these cells to TRAIL-induced apoptosis. TRAIL is a novel cancer therapeutic protein and is currently in Phase I clinical trials. Our findings suggest a strong relationship between TRAIL-induced apoptosis by pemetrexed. This has important clinical implications in considering combination TRAIL-pemetrexed therapy for NSCLC.

Our group and others have demonstrated that activation of the TRAIL receptor apoptotic pathway such as up-regulation of DR5 plays a critical role in inducing apoptosis by certain anticancer agents.[21,22] We found that apoptosis can occur with 36 h of pemetrexed exposure.[23] We showed that DR5 up-regulation started at 36 h and remained high for 48 h. In addition, DR5 inhibition induced by silencing DR5 expression attenuated pemetrexed's ability to induce apoptosis. This demonstrates that the DR5 pathway is instrumental for pemetrexed-induced apoptosis.

DR5 is known to be regulated by p53,[24,25] nuclear factor-κB,[26], Sp1[27] and CHOP.[28] Because pemetrexed induced DR5 expression in H157 and H1792 cells which carry mutant p53, it is likely that pemetrexed induced a p53-independent DR5 expression. In an effort to discover the mechanistic underpinnings for increased DR5 expression, we found that CHOP was affected by pemetrexed and accompanied with DR5 up-regulation. Moreover, inhibition of CHOP induction abolished pemetrexed induced DR5 expression. Thus, we conclude that pemetrexed triggers a CHOP dependent DR5 up-regulation. Because CHOP is a typical protein associated with endoplasmic reticulum (ER) stress induced apoptosis,[28] it is possible that pemetrexed up-regulation of CHOP and DR5 is caused by ER stress. We also found Bip and IRE1α expression levels to be increased in a concentration-dependent manner, suggesting pemetrexed treatment results in ER stress in lung cancer cells. There is an alternative hypothesis. Since inhibition of thymidylate synthase by pemetrexed shuts off DNA synthesis and triggers cell cycle arrest and apoptosis,[29] we postulate that impaired DNA synthesis may induce the expression of CHOP which, in turn, leads to DR5 up-regulation. Future studies need to elucidate how pemetrexed increases CHOP expression and whether other transcriptional factors are activated and involved in pemetrexed-induced DR5 expression.

Given the important roles of c-FLIP in the regulation of the TRAIL-mediated extrinsic apoptotic pathway and that both DR5 and c-FLIP are strongly modulated by pemetrexed, we hypothesise that DR5 induction and c-FLIP down-regulation are two pivotal events that mediate apoptosis induction by pemetrexed. This is further supported by our discovery that enforced overexpression of ectopic c-FLIP$_L$ confers cell resistance to induction of apoptosis by pemetrexed or the combination of pemetrexed and TRAIL. Although both isoforms of c-FLIP (c-FLIP$_L$ and c-FLIP$_S$) are inhibitors of caspase 8, c-FLIP$_L$ appears to be more potent than c-FLIP$_S$ in protecting cells from apoptosis following chemotherapeutic agents.[19] Thus, we focused on the role of c-FLIP$_L$. We noted that FLIP$_L$ levels were increased by pemetrexed at early time points (e.g. 12 h) and then decreased after prolonged treatment (e.g. 24–48 h). The mechanism and biological significance of the early increase in FLIP$_L$ remain unclear. We speculate that the early rise in FLIP$_L$ levels may represent a survival mechanism for cells attempting to escape pemetrexed-induced cell death.

We have noted that the cleaved band of PARP (a known substrate of caspases) does appear earlier (24 h) in our data (Figs. 2B, 4B, 5B) than up-regulation of CHOP and DR5 levels or caspase cleavage. This finding may be explained by two possibilities: First, the cleavage of PARP at 24 h was minimal, and the band may be due to the high affinity of the PARP antibody and long exposure time when the Western Blot assay was performed. Second, it is possible that there was weak PARP cleavage at an earlier time point, and this may be due to weak necrotic cell death induced by PEM which was independent of caspase activation. Regardless, the most dramatic cleavage of PARP occurs at 36 h. CHOP and DR5 levels are fully up-regulated at 24 and 36 h and the cleaved caspases increase at 36 h. This correlates with PEM induced apoptosis after 36 h.[7]

Considering pemetrexed has been in clinical trials in several cancers, our study may have important clinical implication. In previous studies, patients have received 400–700 mg/m^2 of pemetrexed as a 10-min infusion on day 1 of a 21-d cycle. Their maximum plasma concentration of pemetrexed is 89.1 ± 25.2 to 131 ± 18.4 μg/ml (equivalent to 208.5 ± 59.0 to 306.5 ± 43.0 μmol/L).[30] Pemetrexed is eliminated rapidly from the plasma with a elimination half-life ($t_{1/2}$) of 2 and 5 h at doses ranging from 525 to 700 mg/m^2.[31] Our study shows pemetrexed induces apoptosis in lung cancer cells at 2.5–10 μmol/L for relatively longer time (48 h). Our data suggest that if we could prolong the time interval that pemetrexed remains in the tumour, despite the fact that the local concentration would be lower, there would be sufficient drug levels to induce apoptosis in cancer cells. The advantage of this paradigm is the drug efficacy may be better while being less toxic.

In summary, this study demonstrates for the first time that pemetrexed induces CHOP-mediated DR5 up-regulation and down-regulation of c-FLIP, leading to apoptosis and the enhancement of TRAIL-induced apoptosis in human NSCLC cells. This has both mechanistic and translational implications to future treatment strategies for patients with NSCLC.

Role of the funding source

Test materials, equipment, testing, etc. are supported by the fund.

Conflict of interest statement

None declared.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (30971479 and 31071215), Doctoral Fund of Ministry of Education of China (20090131110002), Independent Innovation Foundation of Shandong University (IIFSDU 2009JQ006), Shandong Natural Science Foundation (BS2009YY004, 2010GSF10218 and JQ201007).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.06.003.

REFERENCES

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin* 2010;**60**:277–300.
2. Cohen MH, Justice R, Pazdur R. Approval summary: pemetrexed in the initial treatment of advanced/metastatic non-small cell lung cancer. *Oncologist* 2009;**14**:930–5.
3. Jackson RC. Biological effects of folic acid antagonists with antineoplastic activity. *Pharmacol Ther* 1984;**25**:61–82.
4. Allegra CJ. Antifolates. in: Chabner BA, Collins JM (eds.), Cancer Chemotherapy: Principles & Practice. Lippincott: Philadelphia; 1990. p. 110–153.
5. Schultz RM. Newer antifolates in cancer therapy. In: Jucker E, editor. *Progress in Drug Research*, vol. 44. Basel: Birkhauser Verlag; 1995. p. 129–57.
6. McLeod HL, James C, Robert HP, et al. Pharmacokinetic and Pharmacodynamic Evaluation of the Glycinamide Ribonucleotide Formyltransferase Inhibitor AG2034. *Clin Cancer Res* 2000;**6**:2677–84.
7. Fisher TC, Milner AE, Gregory CD, Jackman Al, Aherme GW. Bcl-2 modulation of apoptosis induced by anticancer drugs: resistance to thymidylate stress is independent of classical resistance pathways. *Cancer Res* 1993;**53**:3321–8.
8. Ramirez JM, Ocio EM, San Miguel JF, Pandiella A. Pemetrexed acts as an antimyeloma agent by provoking cell cycle blockade and apoptosis. *Leukemia* 2007;**21**:797–804.
9. Lu X, Errington J, Curtin NJ, Lunec J, Newell DR. The impact of p53 status on cellular sensitivity to antifolate drugs. *Clin Cancer Res* 2001;**7**:2114–23.
10. Cotter T. G. Apoptosis and cancer: the genesis of a research field. *Nat Rev Cancer* 2009;**9**:501–7.
11. Ashkenazi A. Dixit VM Death receptors: signaling and modulation. *Science* 1998;**281**:1305–8.
12. Whiteside TL. The role of death receptor ligands in shaping tumor microenvironment. *Immunol Invest* 2007;**36**:25–46.
13. Liu X, Yue P, Zhou Z, Khuri FR, Sun SY. Death receptor regulation and celecoxib-induced apoptosis in human lung cancer cells. *J Natl Cancer Inst* 2004;**96**:1769–80.
14. Nagane M, Pan G, Weddle JJ, et al. Increased death receptor 5 expression by chemotherapeutic agents in human gliomas causes synergistic cytotoxicity with tumor necrosis factor-related apoptosis-inducing ligand in vitro and in vivo. *Cancer Res* 2000;**60**:847–53.
15. Gibson SB, Oyer R, Spalding AC, Anderson SM, Johnson GL. Increased expression of death receptors 4 and 5 synergizes the apoptosis response to combined treatment with etoposide and TRAIL. *Mol Cell Biol* 2000;**20**:205–12.
16. Irmler M, Thome M, Hahne M, et al. Inhibition of death receptor signals by cellular FLIP. *Nature* 1997;**388**:190–5.
17. Kataoka T. The caspase-8 modulator c-FLIP. *Crit. Rev Immunol.* 2005;**25**:31–58.
18. Chen S, Liu X, Yue P, et al. CHOP-dependent DR5 induction and ubiquitin/proteasome-mediated c-FLIP downregulation contribute to enhancement of TRAIL-induced apoptosis by dimethyl-celecoxib in human non-small cell lung cancer cells. *Mol Pharmacol* 2007;**72**:1269–79.
19. Liu X, Yue P, Schonthal AH, Khuri FR, Sun SY. Cellular FLICE-inhibitory protein down-regulation contributes to celecoxib-induced apoptosis in human lung cancer cells. *Cancer Res* 2006;**66**:11115–9.
20. Oh YT, Liu X, Yue P, et al. ERK/RSK signaling positively regulates death receptor 5 expression through co-activation of CHOP and Elk1. *J Biol Chem* 2010;**285**:41310–9.
21. Elrod HA, Sun SY. Modulation of death receptors by cancer therapeutic agents. *Cancer Biol Ther* 2008;**7**:163–73.
22. Sun SY. Chemopreventive agent-induced modulation of death receptors. *Apoptosis* 2005;**10**:1203–10.
23. Tonkinson JL, Marder P, Andis SL, Schultz RM, Gossett LS. Cell cycle effects of antifolate antimetabolites: implications and cytostasis. *Cancer Chemother Pharmacol* 1997;**39**:521–31.
24. Takimoto R, El-Deiry WS. Wild-type p53 transactivates the KILLER/DR5 gene through an intronic sequence-specific DNA-binding site. *Oncogene* 2000;**19**:1735–43.
25. Wu GS, Burns TF, McDonald 3rd ER, et al. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nat Genet* 1997;**17**:141–3.
26. Shetty S, Graham BA, Brown JG, et al. Transcription factor NF-kappaB differentially regulates death receptor 5 expression involving histone deacetylase 1. *Mol Cell Biol* 2005;**25**:5404–16.
27. Higuchi H, Grambihler A, Canbay A, Bronk SF, Gores GJ. Bile acids up-regulate death receptor 5/TRAIL-receptor 2 expression via a c-Jun N-terminal kinase-dependent pathway involving Sp1. *J Biol Chem* 2004;**279**:51–60.
28. Yamaguchi H, Wang HG. CHOP is involved in endoplasmic reticulum stress-induced apoptosis by enhancing DR5 expression in human carcinoma cells. *J Biol Chem* 2004;**279**:45495–502.
29. Takezawa K, Okamoto I, Tsukioka S, et al. Identification of thymidylate synthase as a potential therapeutic target for lung cancer. *Br J Cancer.* 2010;**103**:354–61.
30. Li KM, Rivory LP, Clarke SJ. Pemetrexed pharmacokinetics and pharmacodynamics in a phase I/II study of doublet chemotherapy with vinorelbine: implications for further optimisation of pemetrexed schedules. *Br J Cancer* 2007;**97**:1071–6.
31. Joerger M, Omlin A, Cerny T, Früh M. The role of pemetrexed in advanced nonsmall-cell lung cancer: special focus on pharmacology and mechanism of action. *Curr Drug Targets.* 2010;**11**:37–47.