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Inhibition of MEK/ERK activation attenuates autophagy and potentiates pemetrexed-induced activity against HepG2 hepatocellular carcinoma cells

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ABSTRACT

Identification of efficient chemo-therapeutic/chemo-preventive agents for treatment of hepatocellular carcinoma (HCC) is important. In this study, we examined the activity of pemetrexed, an anti-folate chemotherapy drug, against HepG2 human HCC cells. Pemetrexed treatment *in vitro* exerted weak but significant cytotoxic activity against HepG2 cells. When analyzing the possible pemetrexed-resistance factors, we indentified that pemetrexed treatment in HepG2 cells induced cyto-protective autophagy activation, evidenced by GFP–light chain 3B (LC3B) puncta formation, p62 downregulation and Beclin-1/LC3B-II upregulation. Correspondingly, autophagy inhibitors, including bafliomycin A1, 3-methyladenine and chloroquine, enhanced pemetrexed-induced cytotoxicity against HepG2 cells. Further, RNAi-mediated knockdown of Beclin-1 in HepG2 cells also increased pemetrexed sensitivity. Pemetrexed activated MEK (mitogen-activated protein kinase/ERK kinase)/ERK (extracellular-signal-regulated kinase) signaling in HepG2 cells, which was required for autophagy induction. Pharmacological inhibition of MEK/ERK activation attenuated pemetrexed-induced autophagy, enhanced HepG2 cell death and apoptosis. In summary, pemetrexed activates MEK/ERK-dependent cyto-protective autophagy, and inhibition of this pathway potentiates pemetrexed's activity in HepG2 cells.

1. Introduction

Hepatocellular carcinoma (HCC), the main liver cancer, is a global health threat and the third-leading cause of cancer-related mortalities worldwide [1]. The estimated HCC annual occurrence was more than half-million, causing over 600,000 deaths annually [1]. The fast majority of HCCs are hard to detect at early stages, and surgical resection is thus very difficult [2]. Meanwhile, many advanced HCCs are resistant to almost all known clinically used chemotherapy drugs [3]. These are all important reasons for searching of novel chemo-therapeutic/chemo-preventive agents for HCC [3].

As an anti-folate drug, pemetrexed exerts its anti-cancer activity through inhibiting enzymes that are essential for synthesis of purine and pyrimidine, which are required for DNA/RNA synthesis and cell growth [4,5]. These pemetrexed-targeted enzymes include thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT) [4,5]. Clinically, pemetrexed is approved by FDA for treatment of non small cell lung cancer (NSCLC) and malignant pleural mesothelioma, among others [6–8]. Meanwhile, pre-clinical studies were performed to test its activity in other cancer cells both *in vivo* and *in vitro* [6–8].

This study first examined the activity of pemetrexed against HepG2 cells *in vitro*. We mainly studied the potential resistance factors of pemetrexed, and focused on the role of MEK (mitogen-activated protein kinase/ERK kinase)/ERK (extracellular-signal-regulated kinase) signaling and autophagy in pemetrexed's actions in HepG2 cells.

2. Materials and methods

2.1. Chemicals and antibodies

PD98059, U0126, 3-methyladenine (3-MA), Bafilomycin A1 (Baf-A1) and chloroquine were purchased from Sigma Chemicals

Abbreviations: 3-MA, 3-methyladenine; Baf A1, Bafilomycin A1; Cq, chloroquine; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular-signal-regulated kinase; GFP, green fluorescence protein; MEK, mitogen-activated protein kinase/ERK kinase; LC-3, light chain 3; HCC, hepatocellular carcinoma.

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(St. Louis, MO). Pemetrexed and MEK-162 was purchased from Selleck.cn (Beijing, China). Anti-ERK1/2, MEK1/2, p62 and Beclin-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other antibodies utilized in this study were purchased from Cell Signaling Technology (Bevery, MA).

2.2. HepG2 cell culture

Human HCC HepG2 cells, purchased from Shanghai Institute of Biological Science Cell Bank (Shanghai, China), were grown in DMEM-F12 medium (GIBCO/BRL, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS, GIBCO/BRL) and the following antibiotics: gentamicin, penicillin, and streptomycin (50 mg/L) (All from GIBCO/BRL). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. MTT assay

Cell viability was tested by MTT assay. Briefly, following treatment, medium was removed and replaced with fresh medium (100 μl/well). A total of 10 μl stock MTT (10 mg/ml, Sigma) was added to each well and cells were incubated for 1 h at 37 °C. Medium was then removed. Cells were lysed and formazan was solubilized with DMSO (100 μl/well). Absorbance was measured at 490 nm using a multiwell scanning spectrophotometer (Molecular Devices, Sunnyvale, CA).

2.4. Clonogenic assay

HepG2 cells were seeded in 60-mm tissue culture dishes and allowed to attach overnight. Cells were then incubated in a humidified incubator at 37 °C and 5% CO_2 until there were at least 50 cells per colony. These colonies were then incubated with drug containing medium (refreshed every 2 days) for a total of 10 days. At the end of the incubation, cells were washed with phosphate-buffered saline (PBS, GIBCO/BRL) and incubated with a 0.25% crystal violet solution for 15 min. Following washes with PBS, colonies were counted using the colony-counting tool in the BioChemi System (UVP, Upland, CA).

2.5. Quantification of apoptosis by DNA fragmentation detection assay (ELISA)

Quantitative determination of cytoplasmic histone-DNA fragments indicative of apoptosis was performed by enzyme-linked immunosorbent assay (ELISA) using the Cell Apoptotic Death Detection ELISA kit (Roche, Indianapolis, IN). Cells were seeded in a 96-well plate (1×10^4/well). After treatment, both adherent and floating cells were lysed and incubated with mouse monoclonal anti-histone-biotin antibody and mouse monoclonal anti-DNA-peroxidase in streptavidin-coated microtiter plates. Unbound antibodies were washed out. The amount of nucleosome was determined quantitatively by evaluating peroxidase activity photometrically (450 nm) with 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid; ABTS) as the substrate.

2.6. Western blot analysis

Equal amounts of proteins (25–40 μg) were separated by SDS–polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride filters (PVDF, Protein Sequencing Membrane, Bio-Rad) that were probed with the corresponding primary antibody. Western blots were developed with the ECL system (GE Healthcare). Bands were quantified by laser scanning densitometry (Molecular Dynamics).

2.7. Quantification of autophagic cells

The protocol was based on Min et al. [9]. HepG2 cells were transfected with GFP (green fluorescence protein)–light chain 3 (LC3) pcDNA3 plasmid (a gift from Dr. Min [9]) using Lipofectamine 2000 (Invitrogen, USA) in serum- and antibiotic-free medium for 6 h, followed by a 72 h incubation in growth medium (with 1% FBS). Afterwards, cells were selected with 1 mg/ml G-418 (GIBCO) to establish a stable cell line expressing the GFP–LC3 fusion protein. Selected cells were seeded onto confocal cover-slips and treated as described. The accumulation of GFP–LC3 was examined by fluorescence microscopy. Autophagic cells were recorded by counting the percentage of cells showing an accumulation of intense GFP–LC3 puncta, analyzing 100 cells per preparation in five independent experiments.

2.8. Beclin-1 RNA interference (RNAi)

To knockdown of Beclin-1, HepG2 cells were transfected with the commercially obtained Beclin-1 siRNAs. Two Beclin-1 siRNAs were from Cell Signaling Tech (-a) and Santa Cruz Biotech (-b) respectively. Control cells were transfected with a scramble control siRNA ("sc-RNAi", Santa Cruz Biotechnology). siRNA (100 nM each) transfection was performed through Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the procedure attached. The transfection took 24 h, and the whole transfection was repeated another round, expression of Beclin-1 was tested by Western blots to confirm RNAi efficiency.

2.9. Statistics analysis

Data were presented as mean ± SEM. Multiple group comparisons were performed using ANOVA with a post hoc test (SPSS, 13.0). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Pemetrexed treatment exerts weak but significant cytotoxic effect in HepG2 cells

We first tested the activity of pemetrexed *in vitro*. HepG2 cells were treated with indicated concentration (0.1–100 μM) of pemetrexed, and its activity was analyzed. MTT assay in Fig. 1A showed that high concentrations of pemetrexed at (10/100 μM) only slightly inhibited HepG2 cell survival, resulting in less than 15–25% of viability reduction after 72 h of treatment. Results from the clonogenic assay showed similar results, and treatment of pemetrexed (10/100 μM) for ten consecutive days reduced the number of survival colonies (Fig. 1B). Further, pemetrexed administration (10–100 μM, 72 h) only induced minor but significant apoptosis in HepG2 cells (Fig. 1C). These results indicate that pemetrexed alone, even at high concentrations, only exerts weak cytotoxic effect against HepG2 cells.

3.2. Pemetrexed activates cyto-protective autophagy in HepG2 cells, counteracting cell death

Above results demonstrated that pemetrexed only exerted weak activity against HepG2 cells. Next, we studied the potential resisting factors. Autophagy could be activated by a number of anti-cancer drugs, which functions as a cyto-protective factor. While autophagy inhibition given in combination with these drugs could promote chemo-sensitization [10–14]. We thus tested autophagy activation in pemetrexed-treated HepG2 cells. Western

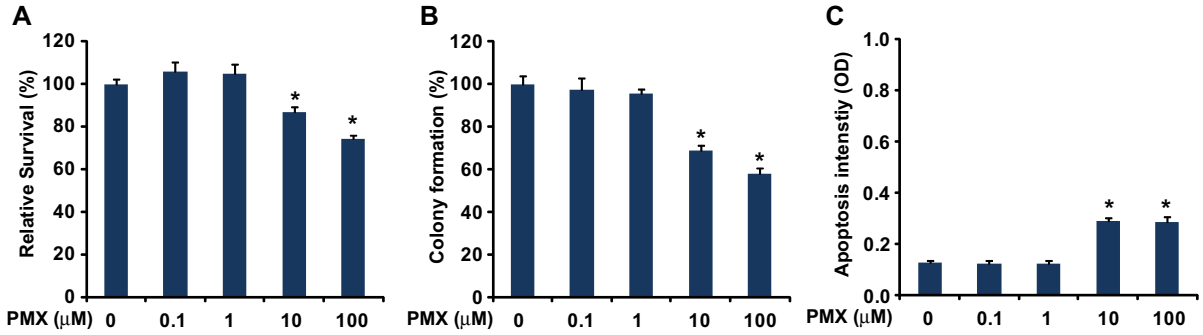

Fig. 1. Pemetrexed treatment exerts weak but significant cytotoxic effect in HepG2 cells. HepG2 cells with indicated pemetrexed ("PMX") treatment were analyzed by MTT assay (A, 72 h), clonogenic assay (B, 10 days) and Histone-DNA apoptosis ELISA assay (C, 72 h). Values in (A) and (B) were normalized to pemetrexed (0 μM) group. Data were shown as mean ± SEM ($n = 5$) of one representative experiment (same for all figures). Experiments in this and following figures were repeated four times, with similar results obtained. $^{*}p < 0.05$ vs. pemetrexed (0 μM) group.

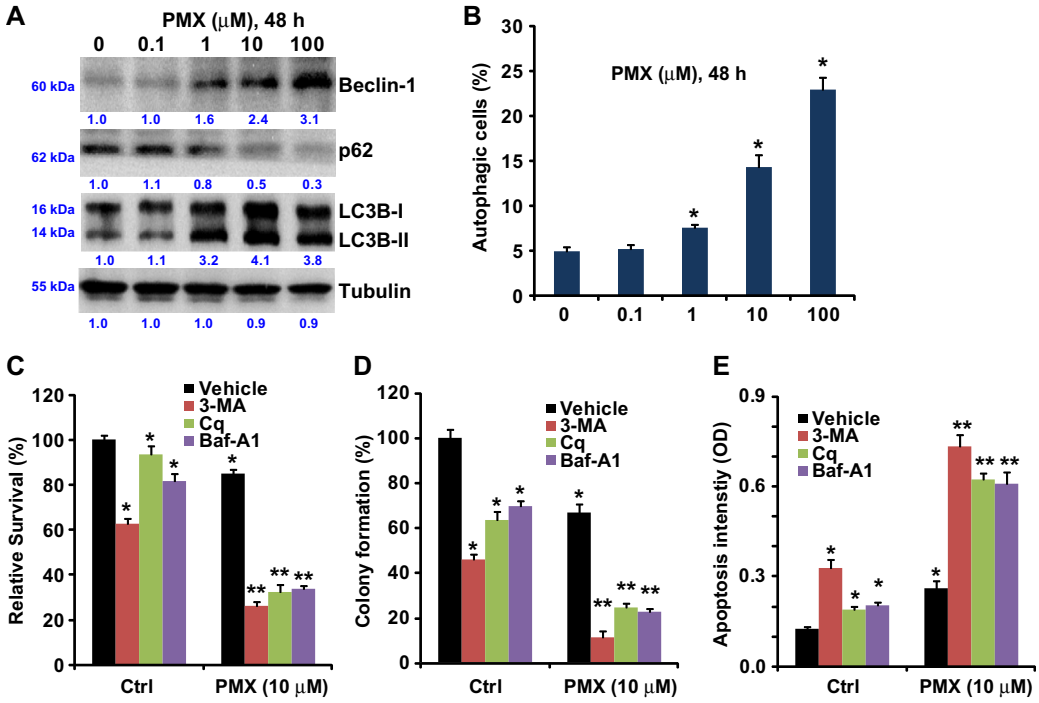

Fig. 2. Pemetrexed activates cyto-protective autophagy in HepG2 cells, resisting cell death. HepG2 cells with indicated pemetrexed ("PMX") stimulation were analyzed by Western blots to test indicated proteins (A), cells with intense LC3B fluorescence puncta (autophagic cells) were quantified as described (B). HepG2 cells, pre-treated with 3-methyladenine (3-MA, 1 mM), Bafilomycin A1 (Baf-A1, 50 nM) or chloroquine (Cq, 25 μM) for 2 h, were stimulated with pemetrexed (10 μM), cells were then analyzed by MTT assay (C, 72 h), clonogenic assay (D, 10 days) and Histone-DNA apoptosis ELISA assay (E, 72 h) as described. Blot intensity was quantified and normalized to pemetrexed (0 μM) group (A). Vehicle stands for 0.1% DMSO. "Ctrl" stands for vehicle only group. Values in (C) and (D) were normalized to "Ctrl" group. $^{*}p < 0.05$ vs. pemetrexed (0 μM) or "Ctrl" group. $^{**}p < 0.05$ vs. pemetrexed (10 μM) only group.

blot results in Fig. 2A showed that pemetrexed treatment induced p62 downregulation as well as Beclin-1 and LC3B-II upregulation, indicating autophagy induction. Further, the number of HepG2 cells with intense LC3B fluorescence puncta was increased after pemetrexed treatment (Fig. 2B), further confirming autophagy activation in these cells.

To study the potential role of autophagy in pemetrexed's activity. Autophagy inhibitors were applied. Bafliomycin A1 (Baf-A1), 3-methyladenine (3-MA) and chloroquine (Cq), three different autophagy inhibitors (see Section 4), significantly enhanced pemetrexed-induced cytotoxicity, shown by dramatically intensified viability inhibition (Fig. 2C), colony number reduction (Fig. 2D) and apoptosis activation (Fig. 2E) in HepG2 cells. These results suggest that pemetrexed-activated autophagy exerts a cyto-protective role. Notably, as shown in Fig. 2D–F, these autophagy inhibitors alone also induced HepG2 cell death and apoptosis. Thus, un-stimulated basal autophagy appeared also pro-survival in HepG2 cells.

3.3. Beclin-1 RNAi in HepG2 cells increases pemetrexed's activity

To rule out the possible off-target effect of above inhibitors, RNAi method was applied to knockdown Beclin-1, a key autophagy regulator [15], in HepG2 cells. Beclin-1 targeted siRNA significantly downregulated Beclin-1 expression in HepG2 cells (Fig. 3A). Note that two non-overlapping siRNAs (Beclin-1 RNAi-a/-b) against Beclin-1 were applied, and both showed high efficiency in down-regulating Beclin-1 in HepG2 cells (Fig. 3A). In consistent with

the inhibitor results, pemetrexed-induced cytotoxicity and apoptosis were significantly enhanced by RNAi knockdown of Beclin-1 (Fig. 3B and C), further supporting that autophagy inhibition could sensitize the pemetrexed's activity in HepG2 cells.

3.4. MEK/ERK activation is required for autophagy induction by pemetrexed in HepG2 cells

Next, we studied the underlying mechanisms of autophagy activation by pemetrexed in HepG2 cells. It has been shown that pemetrexed activates MEK/ERK signaling in a number of cancer cell lines [16,17]. Meanwhile, MEK/ERK is a known activator of autophagy [18,19]. Thus, we tested the potential role of MEK/ERK signaling in pemetrexed-induced autophagy activation. Western blot results in Fig. 4A showed that pemetrexed activated MEK/ERK signaling in HepG2 cells, as phosphorylated (p-) MEK1/2 (Ser217/221) and p-ERK1/2 (Thr202/Tyr204) were upregulated in pemetrexed-stimulated cells (Fig. 4A). MEK/ERK inhibitors including PD98059, U0126 and MEK-162 blocked ERK1/2 phosphorylation by pemetrexed in HepG2 cells (Fig. 4C). Significantly, pemetrexed-induced autophagy activation, shown by LC3B fluorescence puncta formation (Fig. 4B) as well as Beclin-1 and LC3B-II upregulation (Fig. 4C), was dramatically attenuated by above MEK/ERK inhibitors. These results indicate that MEK/ERK activation is required for autophagy induction by pemetrexed in HepG2 cells.

3.5. MEK/ERK inhibition sensitizes pemetrexed's activity in HepG2 cells

Thus far we have shown that pemetrexed activates autophagy in HepG2 cells, exerting anti-apoptosis and cyto-protective effects. Further, activation of MEK/ERK signaling is required for pemetrexed-induced autophagy activation. At last, we tested the potential role of MEK/ERK activation in pemetrexed-induced cytotoxicity in HepG2 cells. MEK/ERK inhibitors including PD98059, U0126 and MEK-162 all sensitized pemetrexed-induced HepG2 viability reduction (Fig. 4D), and cell death (colonies number reduction, Fig. 4E). While apoptosis activation by pemetrexed was enhanced with pharmacological inhibition of MEK/ERK signaling (Fig. 4F). Note that, these MEK/ERK inhibitors alone inhibited basal autophagy in HepG2 cells (Fig. 4B), and was cytotoxic against HepG2 cells (Fig. 4D–F). Together, these results show MEK/ERK inhibition attenuates autophagy and potentiates pemetrexed-induced cytotoxicity against HepG2 cells.

4. Discussions

Pemetrexed potently inhibits folate synthesis enzymes, thereby depleting nucleotide pools to block DNA synthesis. It is a newly indentified anti-cancer drug, and its efficiency has not been the same among various cancer cell lines [4,5,20]. Here we found that pemetrexed only exerted weak activity against HepG2 cells *in vitro*, partly because it simultaneously activated cyto-protective autophagy. These pre-clinical observations *in vitro* are consistent with clinical findings [21]. Correspondingly, inhibition of autophagy through pharmacological inhibitors or Beclin-1 RNAi significantly increased pemetrexed-induced cytotoxicity against HepG2 cells. We indentified that activation of MEK/ERK signaling is required for pemetrexed-induced autophagy induction in HepG2 cells. MEK/ERK inhibition attenuated pemetrexed-induced autophagy, thus increasing pemetrexed's cytotoxicity in HepG2 cells.

When autophagy is activated, cells clear the damaged proteins or organelles through lysosomal degradation, which supplies energy and nutrients for cell survive [10]. In cancer cells, activation autophagy is considered as a cyto-protective factor [10,22]. Several autophagy inhibitors were utilized in this study. Chloroquine (Cq) is a lysosomotropic drug which inhibits lysosomal degradation through increasing intra-lysosomal pH [23]. The PI3K inhibitor 3-methyladenine (3-MA) stops autophagy initiation [23]. Bafilomycin A1 (Baf-A1) is known to block the fusion of autophagosome with the lysosome [23]. All these inhibitors significantly increased pemetrexed-induced cytotoxicity in HepG2 cells, indicating that autophagy activation by pemetrexed functions as a cyto-protective resistance factor.

It should be noted that all these inhibitors are non-specific inhibitors of autophagy, and have off-targeted effects [23]. The evidence further supporting our hypothesis is that RNAi knockdown of Beclin-1, a key protein required for autophagosome formation, significantly increased the sensitivity of pemetrexed in HepG2 cells. Thus, pemetrexed-activated autophagy counteracts its cytotoxicity in HepG2 cells, while autophagy inhibition through genetic or pharmacologic means significantly increased pemetrexed's sensitivity in HepG2 cells.

Activation of MEK/ERK signaling has been involved in autophagy induction by several stimuli including amino acid deprivation [24], aurintricarboxylic acid [25], B-group soyasaponins [26] and curcumin [27]. Studies have also investigated the signaling mechanism of MEK/ERK in mediating autophagy induction. It has been shown that MEK/ERK stimulates autophagy by regulating Beclin-1 [18]. Partially depletion (through siRNA) or pharmacological inhibition of MEK/ERK signaling inhibited Beclin-1 upregulation and cell autophagy [18]. In this study, we indentified that activation of MEK/ERK is important for pemetrexed-induced Beclin-1 expression as well as autophagy induction. Pharmacological inhibition of MEK/ERK attenuated pemetrexed-induced Beclin-1 upregulation/autophagy activation, and significantly increased its cytotoxicity against HepG2 cells. Thus, pemetrexed-activated

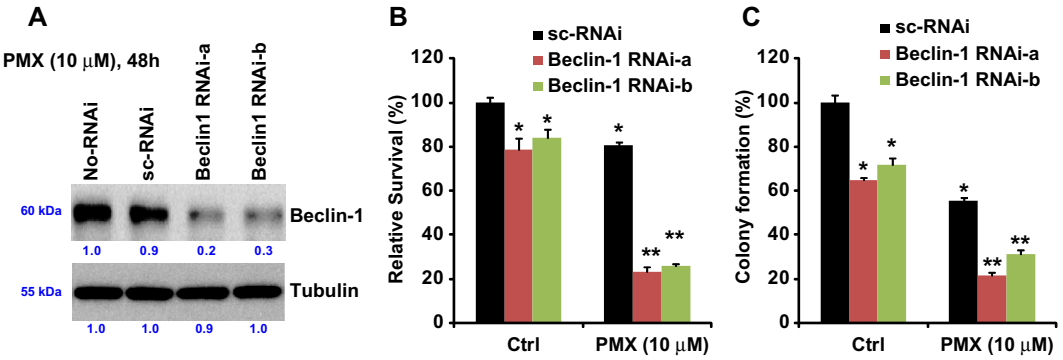

Fig. 3. Beclin-1 RNAi in HepG2 cells increases pemetrexed's activity. Beclin-1 and tubulin (equal loading) expression in HepG2 cells transfected with or without indicated siRNA (100 nM, 24 h, twice) was shown, cells were stimulated with pemetrexed (10 μM) ("PMX") for 48 h (A). HepG2 cells transfected with indicated siRNA were treated with pemetrexed (10 μM), cells were then analyzed by MTT assay (B, 72 h) and clonogenic assay (C, 10 days). Beclin-1 expression was quantified and normalized to "No RNAi" group (A). "Ctrl" stands for no pemetrexed group. $^{*}p < 0.05$ vs. "Ctrl" of "sc-RNAi" group. $^{**}p < 0.05$ vs. pemetrexed (10 μM) of "sc-RNAi" group.

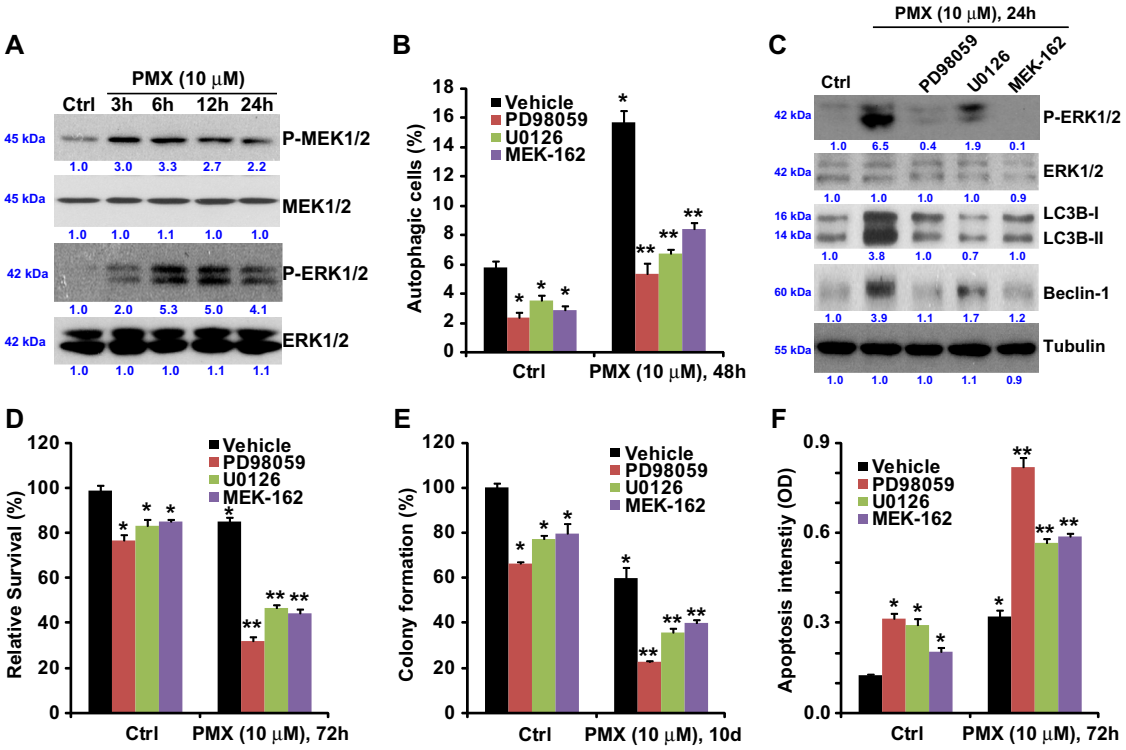

Fig. 4. MEK/ERK inhibition attenuates autophagy and potentiates pemetrexed-induced cytotoxicity against HepG2 cells. MEK/ERK activation in HepG2 cells with indicated pemetrexed ("PMX") treatment was tested (A). HepG2 cells, pre-treated with PD98059 (500 nM), U0126 (500 nM) or MEK-162 (250 nM) for 1 h, were stimulated with pemetrexed (10 μM) for indicated time, autophagic cells were counted through GFP–LC3B puncta (B), expression of indicated protein was tested by Western blot assay (C), cells were also subjected to MTT assay (D, 72 h), clonogenic assay (E, 10 days) and Histone-DNA apoptosis ELISA assay (F, 72 h). Vehicle stands for 0.1% DMSO. "Ctrl" stands for vehicle only group. Blot intensity was quantified and normalized to "Ctrl" group (A and C). $^{*}p < 0.05$ vs. "Ctrl" group. $^{**}p < 0.05$ vs. pemetrexed (10 μM) only group.

MEK/ERK signaling mediates autophagy activation, exerts a cyto-protective role.

In summary, pemetrexed activates MEK/ERK-dependent, cyto-protective autophagy in HepG2 cells, and inhibition of this pathway potentiates pemetrexed's activity.

Conflict of interest

No conflict of interests were stated by authors.

Acknowledgments

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