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Hepatocyte growth factor suppresses the anticancer effect of irinotecan by decreasing the level of active metabolite in HepG2 cells

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ABSTRACT

In the liver, carboxylesterase (CES) converts irinotecan (CPT-11) to its active metabolite SN-38, which exerts anticancer effects. SN-38 is metabolized to an inactive metabolite SN-38 glucuronide by uridine 5′-diphospho-glucuronosyltransferase 1A1 (UGT1A1). Therefore, single nucleotide polymorphisms (SNPs) of the *UGT1A1* gene are responsible for the severe adverse effects associated with the disruption of SN-38 metabolism. However, despite having SNPs of the *UGT1A1* gene, many patients metabolize SN-38 sufficiently to avoid severe adverse effects. Among these patients, we found individuals with elevated serum concentrations of hepatocyte growth factor (HGF). The aim of this study was to evaluate whether HGF alters the metabolism of CPT-11, resulting in a reduction in the anticancer effect of CPT11. The cytotoxicity of CPT-11 and SN-38 was evaluated in HepG2 cells pretreated with HGF. Furthermore, we explored the level of expression and mechanisms of activity of CES and UGT1A1. HGF suppressed the cytotoxicity of CPT-11 by decreasing intracellular SN-38 levels that resulted from a decrease in CES2 and an increase in UGT1A1. Furthermore, this HGF-induced suppression was improved by pretreatment with an inhibitor of HGF receptor c-Met, and the improvement was synergistically potentiated by epidermal growth factor receptor (EGFR) inhibitors. Moreover, HGF induced phosphorylation of signal transducer and activator of transcription 3 and transactivated EGFR. These results suggest that HGF is a possible causative agent of acquired clinical resistance in chemotherapy with CPT-11 and could be useful as a predictor of clinical resistance. Additional treatment using c-Met and/or EGFR inhibitors could be a novel strategy to overcome resistance.

1. Introduction

Hepatocyte growth factor (HGF) and epidermal growth factor (EGF) induce the differentiation, proliferation, and migration of cancer cells. Therefore, HGF is counterproductive to the successful treatment of cancer. HGF has been reported to be elevated in the serum of patients with various malignancies, including non-small cell lung cancer [1,2] and colorectal cancer [3]. In these studies, the percentage of cancer patients with elevated HGF level has been shown to be 55.5% and 53.3% in non-small cell lung cancer and colorectal cancer, respectively. Recently, the serum levels of HGF in patients with hepatocellular carcinoma (HCC) have been reported to be higher than those in the healthy controls, and these levels negatively correlated with the survival time [4]. The association of high serum HGF and rapid cancer progression in HCC is attributable not only to HGF-induced mitogenic effects, but also to other effects of HGF on metabolism and excretion, because hepatocytes have major roles in metabolism and excretion. Therefore, the effects of HGF on metabolic enzymes and transporters have been evaluated. Donato et al. showed that HGF downregulates the expression of cytochrome P450 (CYP) isozymes [5]. We have reported that HGF increases the protein level of organic anion-transporting polypeptide, which is probably involved in the uptake of epirubicin, an antineoplastic [6]. However, although irinotecan (CPT-11) is frequently used for the treatment of cancer patients with high serum concentrations of HGF [1–3], the effects of HGF on the antineoplastic effect of CPT-11 are poorly understood.

In the liver, carboxylesterase (CES) converts CPT-11 to its active metabolite SN-38 (Fig. 1A). However, SN-38 is also responsible for the severe adverse effects, such as neutropenia and diarrhea, which are the dose-limiting factors in the chemotherapy using CPT-11 [7–

Abbreviations: Akt, anti-apoptotic serine–threonine kinase; CES, carboxylesterase; CYP, cytochrome P450; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; Erk, extracellular signal-regulated kinase; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; Jak, Janus kinase; NPA, *p*-nitrophenylacetate; RTK, receptor tyrosine kinase; SNP, single nucleotide polymorphism; STAT3, signal transducer and activator of transcription 3; UGT, uridine 5′-diphospho-glucuronosyltransferase.

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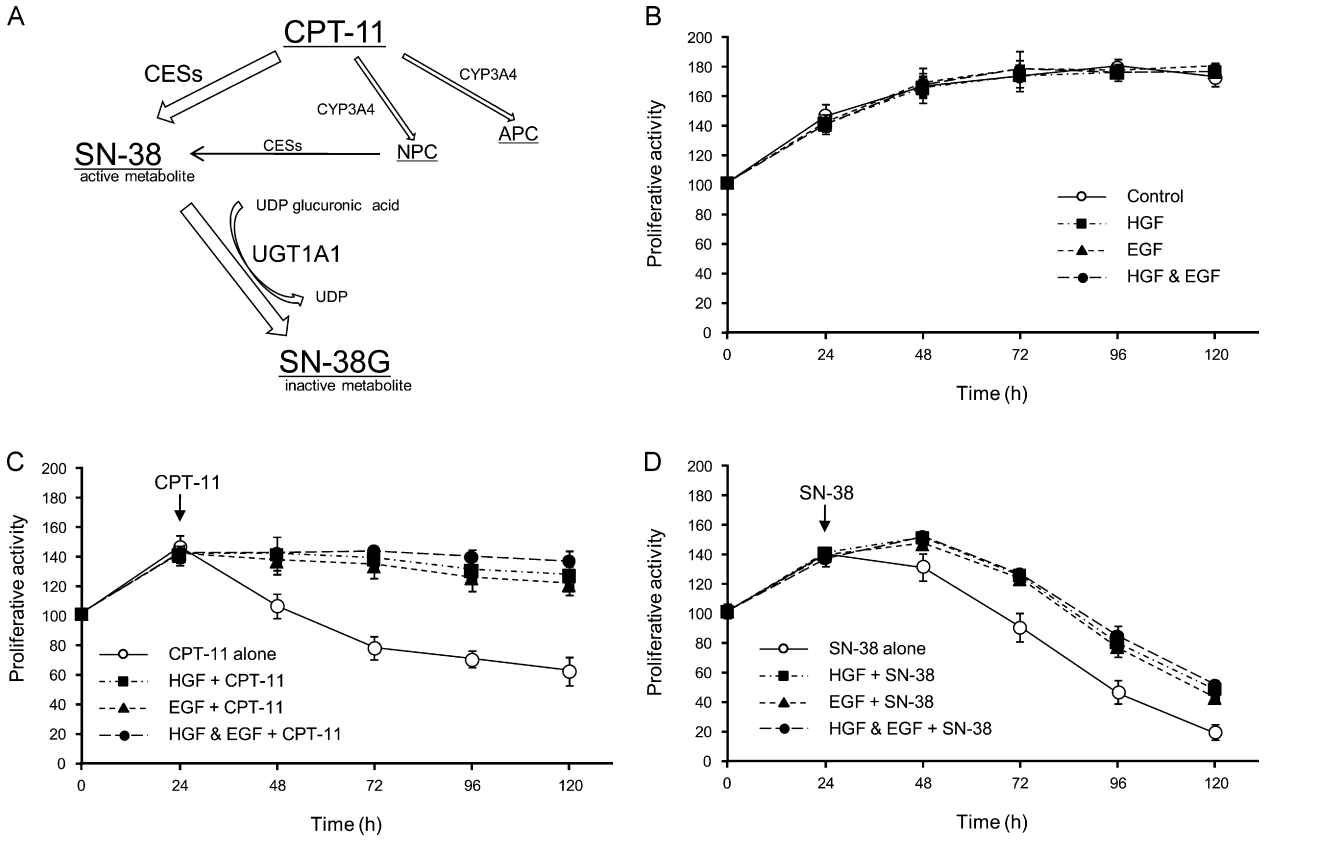

Fig. 1. Metabolism of CPT-11 and the effects of HGF and EGF on the cytotoxicity of CPT-11 and SN-38. Metabolism of CPT-11 is shown in panel (A). HepG2 cells were cultured for 120 h after treatment with HGF (50 ng/mL), EGF (50 ng/mL), or both. Vehicle (B), CPT-11 (10.0 μM) (C), and SN-38 (7.5 μM) (D) were added 24 h after the treatment with HGF and EGF. The proliferative activity was measured at the indicated times and is expressed as a percentage of the proliferative activity in cells not treated with growth factors at 0 h. Each bar represents the mean ± SD of 5 experiments. APC, 7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin; CYP3A4, cytochrome P450 3A4; NPC, 7-ethyl-10-(4-amino-1-piperidino) carbonyloxycamptothecin; UDP, uridine 5′-diphosphate; UGT1A1, UDP-glucuronosyltransferase 1A1; CES, carboxylesterase.

9]. Since, SN-38 is mainly metabolized by uridine 5′-diphosphate-glucuronosyltransferase 1A1 (UGT1A1) to an inactive metabolite SN-38 glucuronide (SN-38G) [10,11], the differences in the expression and activity of UGT1A1 in hepatic tissues have an important influence on the efficacy and cytotoxicity of CPT-11 [12]. Further, these differences have been considered a cause of resistance to CPT-11 [13–15]. At present, the most likely cause of the differences is thought to be single nucleotide polymorphisms (SNPs) in the *UGT1A1* gene. *UGT1A1*28* mutation leads to a partial or complete deficiency of enzyme activity [16,17]. Consequently, patients with *UGT1A1*28* develop severe adverse effects during chemotherapy with CPT-11 [18–20]. However, contrary to the above-mentioned reports, Braun et al. recently reported that *UGT1A1*28* is not associated with the adverse effects of CPT-11 in a large randomized trial [21]. In fact, in some clinical cases, despite the presence of heterozygous *UGT1A1*28*, serum concentration profiles of CPT-11, SN-38, and SN-38G were similar to those of the patients without this SNP. Further, among these cases, there were cases in which the serum level of HGF was significantly higher than normal. In all of these cases, the serum concentration of HGF exceeded 0.4 ng/mL.

On the basis of these findings, we hypothesized that HGF might alter the metabolism of CPT-11, thereby resulting in a reduction in the cytotoxicity of CPT-11. A reduction in cytotoxicity attenuates the severe adverse effects, but leads to a graver prognosis and results in the failure of chemotherapy with CPT-11. Therefore, to validate the hypothesis before beginning a clinical study, we evaluated the influence of HGF on the cytotoxicities of CPT-11 and SN-38 in HepG2 (human cell line derived from hepatocyte carcinoma) cells and explored the possible mechanisms of HGF action.

2. Materials and methods

2.1. Materials

CPT-11, SN-38, and camptothecin were obtained from Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan), and fetal bovine serum was purchased from Invitrogen (Auckland, NZ). HGF, EGF, SU11274, tyrphostin, U0126, A6730, WP1066, and cryptotanshinone were all purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Antibodies were obtained from the following sources and used at the indicated dilutions: GenWay Biotech, Inc. (San Diego, USA), UGT1A1 (1:600); Cell Signaling Technology, Inc. (MA, USA), c-Met, p-c-Met Tyr^{1003}, $Tyr^{1234/1235}$, Tyr^{1349}, EGFR, p-EGFR Tyr^{845}, Tyr^{1045}, Tyr^{1068}, Tyr^{1086}, Tyr^{1148}, Tyr^{1173}, Janus kinase (Jak) 1, p-Jak1, Jak2, p-Jak2, Src, and p-Src (all 1:1000); Santa Cruz Biotechnology (CA, USA), CYP3A4 (1:200), extracellular signal-regulated kinase (Erk) 1/2, p-Erk1/2, anti-apoptotic serine–threonine kinase (Akt) 1/2/3, and p-Akt1/2/3 (all 1:200); Sigma–Aldrich Co. (St. Louis, MO, USA), CES1 (1:800), CES2 (1:200), signal transducer and activator of transcription 3 (STAT3) (1:250), p-STAT3 (1:1000), and α-tubulin (1:6000). Other reagents and biochemicals were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA).

2.2. Cell culture

HepG2 cells from RIKEN BioResource Center (Ibaraki, Japan) were pre-cultured in Minimum Essential Medium supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). The medium was replaced with

fresh serum-free medium supplemented with 25 mM glucose, 90 nM insulin, 20 μM aminolevulinic acid, 1 mM pyruvate, 60 nM Na_2SeO_3, 10 μg/mL human transferrin, and 1 mg/mL linoleic acid-albumin and cultured with or without growth factors (HGF and/or EGF: 50 ng/mL) at 37 °C in 5% CO_2.

2.3. Cell proliferation assay

HepG2 cells were plated in 96-well plates at 3×10^3 cells/well, grown in medium supplemented with 10% fetal bovine serum for 24 h, and were continuously cultured in serum-free medium with the above-mentioned supplements with or without HGF and/or EGF for 24 h. Thereafter, the cells were treated with CPT-11 and SN-38 for the indicated times. Proliferative activity was detected using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega Corporation, WI, USA).

2.4. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

A reverse transcription reaction was performed using the PrimeScript RT reagent kit (Takara Bio, Otsu, Japan). cDNA synthesized from 100 ng of total RNA was added to a PCR mixture containing SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan), one of the primer sets (UGT1A1 [forward primer: 5′-TGG CTG TTC CCA CTT ACT GCA C-3′; reverse primer: 5′-AGG GTC CGT CAG CAT GAC ATC-3′], CES1 [forward primer: 5′-GGT TCA CTG CCT GCG ACA GA-3′; reverse primer: 5′-AGC AGC AGC ATC CCA TCA ATC-3′], and CES2 [forward primer: 5′-GCA GAC CAT GGT GAT GAG CTT C-3′; reverse primer: 5′-CTC GCA AAG TTG GCC CAG TA-3′]), and RNase-free distilled water. PCR was performed using the Thermal Cycler Dice Real-Time System (Takara Bio, Otsu, Japan), and the thermocycling conditions were as follows: incubation for 30 s at 95 °C, 45 cycles of 3 s each at 60 °C, and 30 s at 95 °C.

2.5. Western blot analysis

HepG2 cells, cultured in 6-well plates with or without HGF and/or EGF for 24 h or for the indicated periods, were lysed in 140 μL of RIPA buffer (Sigma–Aldrich, St. Louis, MO, USA). The lysate was centrifuged at 20,000 × *g* for 10 min at 4 °C. Equal amounts of protein extract were separated by electrophoresis using 7–10% polyacrylamide gels containing sodium dodecyl sulfate and were transferred to polyvinyl difluoride membranes. The membranes were then blocked with a blocking solution (5% skim milk or 1% BSA) for 1 h at room temperature and were incubated overnight at 4 °C with primary antibodies. After thorough washing, the membranes were incubated with a secondary antibody for 1 h at 23 °C. The labeled Western blots were visualized using an enhanced chemiluminescence detection system (ECL Plus; GE Healthcare, Bucks, UK).

2.6. Measurement of CPT-11 and SN-38 in HepG2 cells

HepG2 cells were cultured in 24-well plates with or without HGF and/or EGF for 24 h. Then, CPT-11 and SN-38 were added to the medium and incubated for the indicated times. At the end of the incubation period, the cells were washed with ice-cold phosphate buffered saline, and 250 μL of extract solution composed of 0.5 N HCl was added. The cells were kept at 4 °C overnight to lyse the cells completely. The extracts were neutralized by adding 250 μL of 0.5 N NaOH. In order to determine the protein concentration by the method of Lowry et al. [22], 100 μL aliquots of the extract was used and bovine serum albumin was used as the standard. The remaining extracts were centrifuged, and the supernatants were collected as an intracellular sample. A 50 μL sample was mixed with camptothecin, serving as an internal standard, and the 350 μL mobile phase consisted of acetonitrile and ammonium acetate (3:7, pH 4.5). The mixture was centrifuged at 13,000 rpm for 4 min, and 10 μL of the supernatant was injected into a high-performance liquid chromatography system (Prominence UFLC; Shimadzu, Kyoto, Japan) equipped with a Shim-PACK XR ODSII analytical column (3.0 mm × 150 mm; Shimadzu, Kyoto, Japan). The fluorescence detector excitation and emission wavelengths were set at 355 and 515 nm respectively. The concentrations of CPT-11 and SN-38 were quantified from a standard curve prepared from known concentrations of CPT-11 and SN-38, and the concentration was determined by dividing the amount of CPT-11 and SN-38 by the protein concentration of the cell lysate.

2.7. p-Nitrophenylacetate (NPA) hydrolysis

Cytosolic protein was harvested from cells treated with or without HGF, EGF, or HGF + EGF. Ten micrograms of cytosolic protein dissolved in 200 μL of 50 mM Tris buffer (pH 8.0) was added to each well of a 96-well plate. The reaction was started by adding NPA (Sigma–Aldrich, St. Louis, MO, USA), a typical substrate of CES2. The absorbance of *p*-nitrophenol converted by CES2 from NPA was measured at 409 nm at the indicated times.

2.8. Statistical analysis

Data are expressed as the mean ± SD. Differences among groups were assessed using Dunnett's multiple comparisons test or unpaired Student's *t*-test after analysis of variance (ANOVA) was performed. Statistically significant differences are indicated by $P < 0.05$ and $P < 0.01$.

3. Results

3.1. HGF and EGF suppress the cytotoxicities of CPT-11 and SN-38

The proliferative effects of both HGF and EGF on cancer cells [1–3] could mask their effects on the metabolism of CPT-11 and SN-38. Because HepG2 cells do not proliferate in response to HGF [23], these cells were selected as our experimental model. HGF and EGF, either alone or in combination, consistently had no effect on the proliferation of HepG2 cells (Fig. 1B). In contrast, the cytotoxicity of CPT-11 on HepG2 cells was significantly suppressed by pretreatment of the cells with either HGF or EGF ($P < 0.01$), and simultaneous pretreatment with both HGF and EGF resulted in a slight, statistically insignificant, additive suppression (Fig. 1C). Similarly, the cytotoxicity of SN-38 on HepG2 cells was also significantly suppressed by pretreatment with HGF and EGF ($P < 0.01$) (Fig. 1D).

3.2. HGF and EGF decrease CES expression and increase UGT1A1 expression

Western blotting was used to assess the expression of the metabolic enzymes. Further, we investigated the expression of mRNA to assess whether changes in gene expression could be responsible for the observed changes in the protein levels. In cells treated with either HGF or EGF, CES1 protein levels decreased to 79% and 61%, respectively (Fig. 2A), and expression of *CES1* mRNA decreased to 61% and 46%, respectively, of that in control cells 24 h after treatment (Fig. 2B). Further, CES2 protein levels decreased to 75% and 68%, respectively (Fig. 2C), and the expression of *CES2* mRNA decreased to 66% and 53%, respectively, of that in control cells 24 h after treatment (Fig. 2D). Simultaneous treatment with both HGF and EGF additively decreased the CES1 and CES2 protein levels to 58% and 61%, respectively (Fig. 2A and C). Similarly, HGF and EGF cooperatively decreased the expression of *CES1* and *CES2*

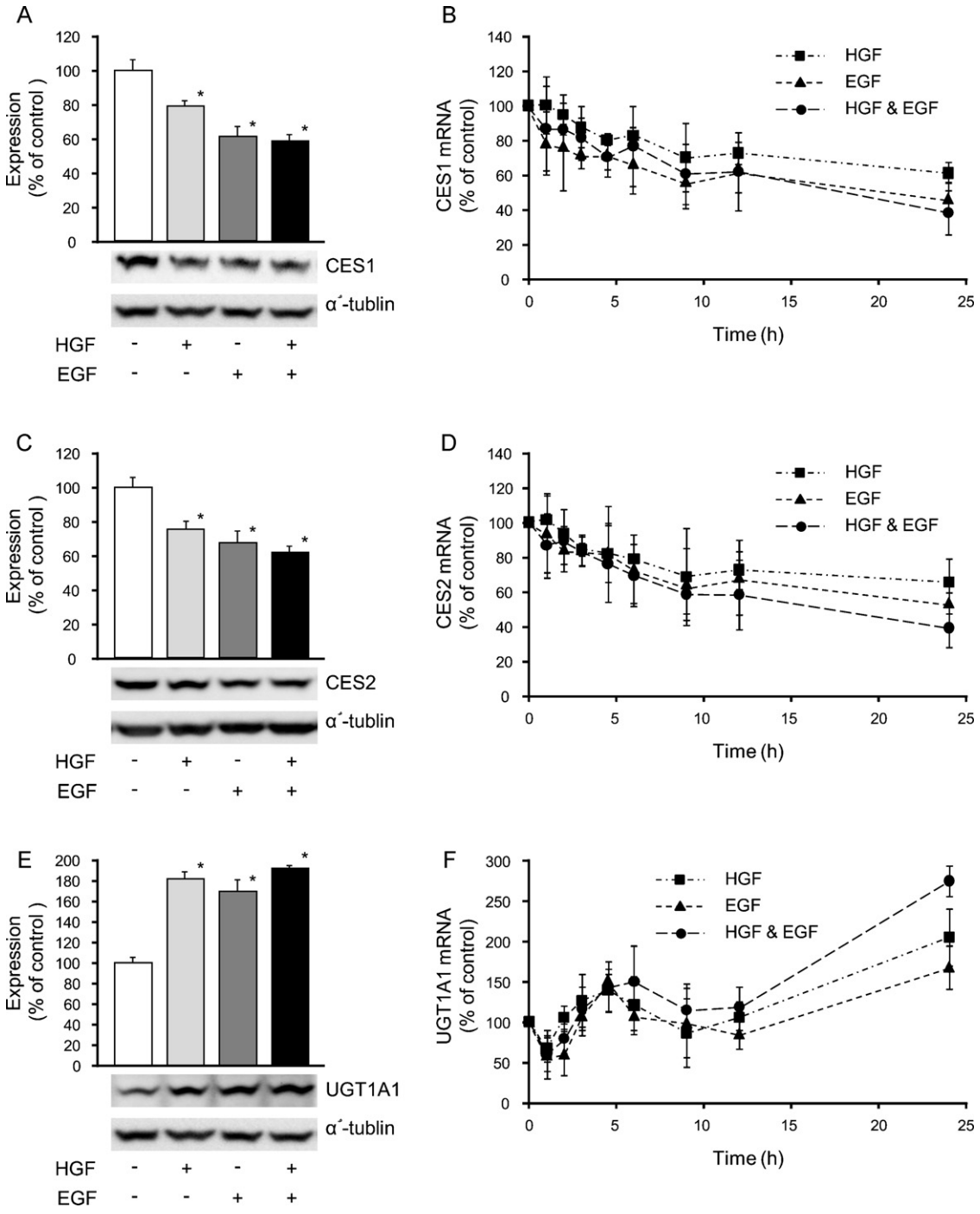

Fig. 2. Effects of HGF and EGF on the protein expression and mRNA of CES and UGT1A1. HepG2 cells were incubated with HGF (50 ng/mL), EGF (50 ng/mL), or both. Total protein was collected 24 h after treatment with growth factors. The expression level of the CES1 (A), CES2 (C), and UGT1A1 (E) proteins was detected by Western blotting. Western blot analysis was performed in 3 experiments for 3 different preparations, and the representative Western blots are shown. The density of the bands was measured using NIH Image-J software. The levels of protein expression quantified by scanning densitometry of the immunopositive proteins and corrected for α-tubulin levels in the same samples are represented as a percentage of that in cells not treated with growth factors. Total RNA were extracted at the indicated times. The transcription levels of *CES1* (B), *CES2* (D), and *UGT1A1* (F) are expressed as a percentage of that in cells not treated with growth factors at 0 h. Each bar represents the mean ± SD. Significant difference from the value in untreated cells: $^{*}P < 0.01$.

mRNA to nearly 40% of that in the control cells (Fig. 2B and D). In contrast, in cells treated with either HGF or EGF, UGT1A1 protein levels increased to 182% and 170%, respectively (Fig. 2E). These results are consistent with those of a previous report [24]. Further, simultaneous treatment with both HGF and EGF additively increased the UGT1A1 protein level to 192%. Similarly, HGF and EGF significantly increased the expression of *UGT1A1* mRNA to 204% and 167%, respectively 24 h after treatment (Fig. 2F), and simultaneous treatment of HGF and EGF additively increased the expression of *UGT1A1* mRNA to 274%.

3.3. HGF and EGF increase intracellular CPT-11 concentration and decrease intracellular SN-38 concentration

The maximum concentrations of CPT-11 in HepG2 cells treated with either HGF or EGF were higher than that in the untreated

control cells, and simultaneous treatment of both HGF and EGF additively increased the intracellular concentration of CPT-11 (Fig. 3A). Similarly, simultaneous treatment of HGF and EGF significantly increased the intracellular area under the curve (AUC) of CPT-11 to 177% (Fig. 3B). In contrast, the concentration of SN-38 in cells treated with either HGF or EGF was lower than that in untreated control cells, and simultaneous treatment of HGF and EGF cooperatively decreased the intracellular concentration of SN-38 (Fig. 3C). Treatment of HepG2 cells with HGF and EGF, either alone or in combination, significantly decreased the AUC of SN-38 to 69%, 63%, and 48%, respectively, of the levels in control cells (Fig. 3D).

3.4. CES2 converts CPT-11 to SN-38 mainly in cells treated with HGF and EGF

NPA hydrolysis was significantly decreased by cytosolic proteins harvested from cells treated with HGF + EGF (Fig. 4A), whereas NPA hydrolysis was slightly decreased by cytosolic proteins harvested from cells treated with HGF or EGF alone. Further, the expression level of the CYP3A4 protein was slightly decreased by treatment with growth factors (Fig. 4B). These results indicate that CPT-11 is mainly converted by CES2 to SN-38 in the cells treated with growth factors.

3.5. Receptor tyrosine kinase (RTK) inhibitors synergistically improve suppression of the cytotoxicities of CPT-11 induced by HGF and EGF

We investigated the effects of RTK inhibitors on growth factor-induced recovery of proliferative activity promoted by the attenuation of CPT-11. The HGF-induced recovery of proliferative activity in CPT-11-treated HepG2 cells was decreased by SU11274, a c-Met inhibitor, and tyrphostin, an EGFR inhibitor (Fig. 5). Moreover, simultaneous treatment of SU11274 and tyrphostin synergistically and completely decreased the recovered proliferative activity. In cells treated with EGF, the recovered proliferative activity was partially but significantly decreased by SU11274, whereas tyrphostin eliminated the recovered proliferative activity. In contrast, the recovered proliferative activity induced by the simultaneous treatment of both HGF and EGF was significantly decreased by tyrphostin, whereas SU11274 had no effect on the recovered proliferative activity. We performed the same experiment with clinically used EGFR inhibitors (gefitinib and erlotinib) and obtained similar results (Supplementary Fig. S1).

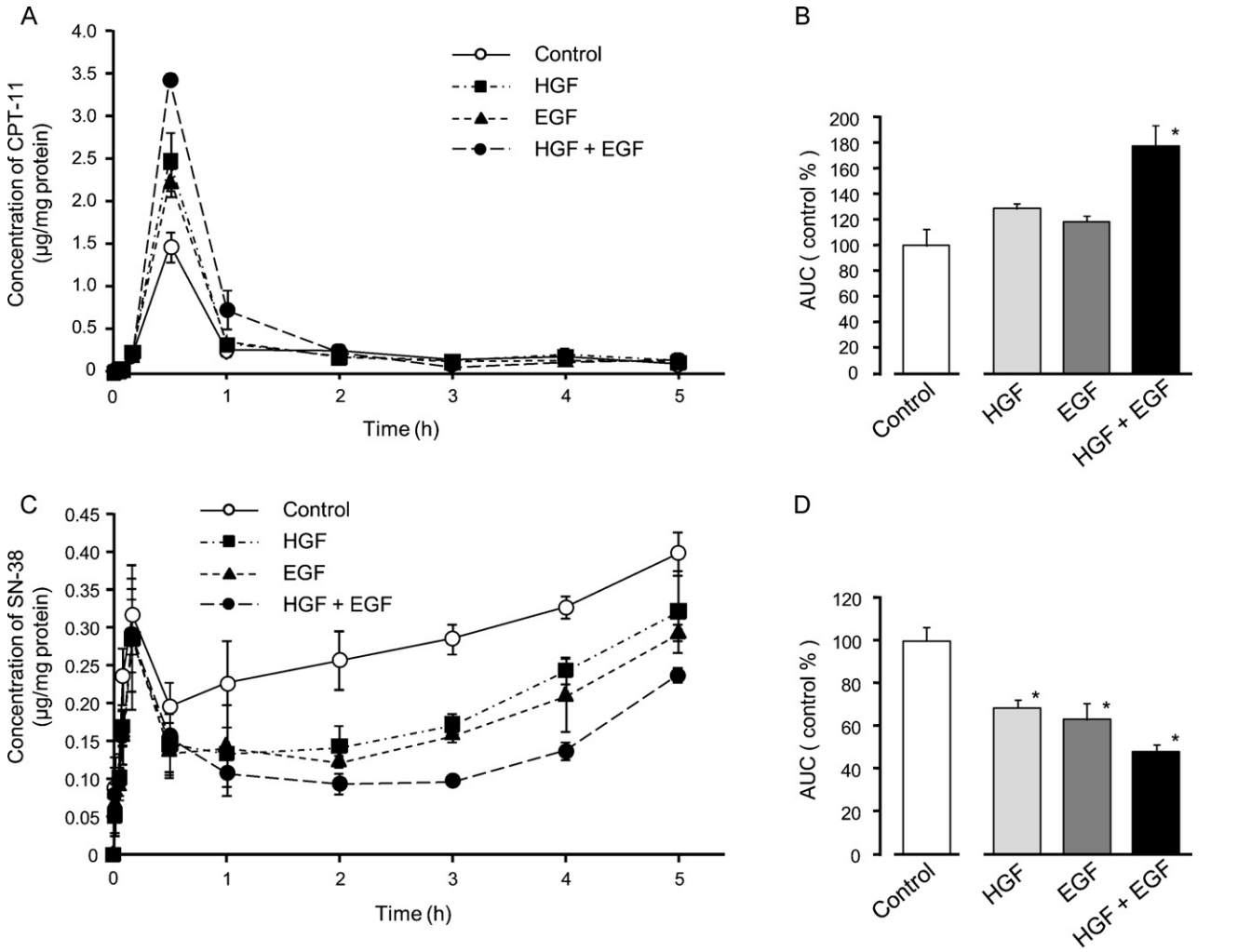

Fig. 3. Effects of HGF and EGF on the intracellular concentration kinetics of CPT-11 and SN-38. CPT-11 (10.0 μM) and SN-38 (7.5 μM) were added to cultures of HepG2 cells 24 h after treatment with HGF (50 ng/mL) and EGF (50 ng/mL). The intracellular CPT-11 (A) and SN-38 (C) were collected at the indicated times, and the concentration was determined by dividing the amount of CPT-11 and SN-38 by the protein concentration of the cell lysate. The AUCs for CPT-11 (B) and SN-38 (D) were calculated according to a linear trapezoidal rule and expressed as a percentage of that in cells not treated with growth factors (control). Each bar represents the mean ± SD of 5 experiments. Significant difference from the value in untreated cells: $^{*}P < 0.01$.

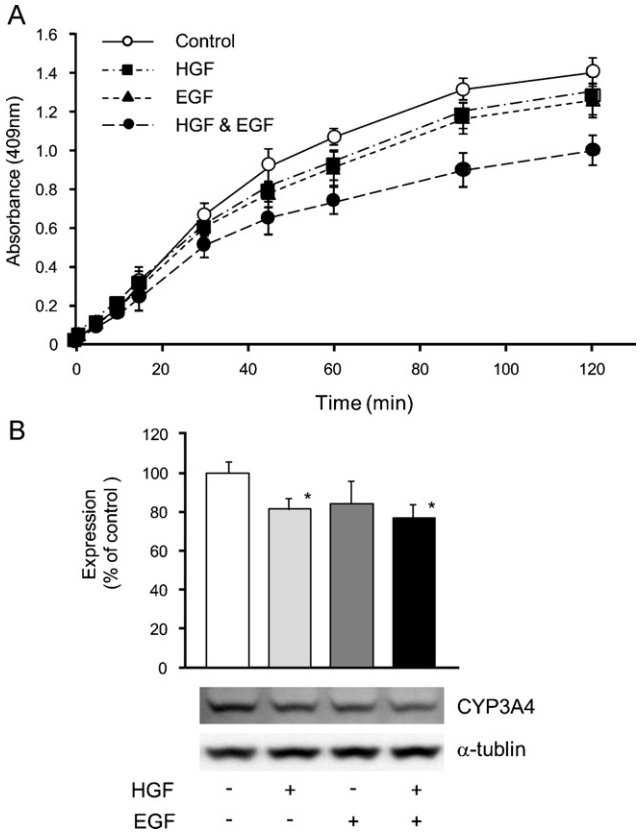

Fig. 4. Confirmation of metabolic enzyme mainly involved in the metabolism of CPT-11. (A) Cytosolic proteins harvested from cells treated with HGF and EGF decrease hydrolysis of NPA. The catalytic activity of CES2 is expressed as absorbance, indicating the amount of *p*-nitrophenol produced by CES2 from NPA. The absorbance was measured at the indicated times. Each bar represents the mean ± SD of 5 experiments. (B) Influence of HGF and EGF on the expression of CYP3A4. Total protein was collected 24 h after treatment with HGF (50 ng/mL) and EGF (50 ng/mL) either alone or in combination and was subjected to Western blotting with specific antibodies for CYP3A4. Western blot analysis was performed in 3 experiments for 3 different preparations, and representative Western blots are shown. The density of the bands was measured using NIH Image-J software. The levels of protein expression quantified by scanning densitometry of the immunopositive proteins and corrected for α-tubulin levels in the same samples are represented as a percentage of that in cells not treated with growth factors. Each bar represents the mean ± SD. Significant difference from the value in untreated cells: $^{*}P < 0.05$.

3.6. Transactivation of phosphorylation sites of EGFR and c-Met by HGF and EGF

Western blotting was performed to detect the phosphorylation of c-Met and EGFR in HGF- and EGF-treated cells and to clarify the mechanism by which HGF- and EGF-induced suppression of CPT-11 cytotoxicity is involved in the crosstalk between these receptors. In cells treated with HGF, the c-Met phosphorylation sites at Tyr^{1003}, $Tyr^{1234/1235}$, and Tyr^{1349} and the EGFR phosphorylation sites at Tyr^{1068}, Tyr^{1086}, and Tyr^{1173} were phosphorylated (Fig. 6A). Although the EGFR Tyr^{1173} phosphorylation induced by EGF was immediate and transient, it was drastically increased after 3 h and peaked at 5 h after treatment of HGF. Further, in cells treated with EGF, c-Met was phosphorylated at the Tyr^{1003} and Tyr^{1349} sites (Fig. 6B). In contrast to the c-Met phosphorylation induced by HGF, the phosphorylation at the Tyr^{1003} site was sustained for the observation period after treatment with EGF, and phosphorylation at the $Tyr^{1234/1235}$ site was not detected. However, the profile of Tyr^{1349} phosphorylation was similar to that of HGF-induced phosphorylation. Simultaneous treatment of cells with both HGF and EGF induced phosphorylation of EGFR at the Tyr^{1068} and Tyr^{1086} sites, and this phosphorylation was stronger than that induced by EGF alone. Simultaneous treatment also induced c-Met phosphorylation at the $Tyr^{1234/1235}$ site, which was not phosphorylated by EGF alone. The time profile of the phosphorylation of other c-Met and EGFR phosphorylation sites after simultaneous treatment was similar to that in cells treated with EGF alone (Fig. 6C).

3.7. HGF drastically increases STAT3 phosphorylation

To elucidate the mechanism of crosstalk between c-Met and EGFR, we investigated the phosphorylation of downstream signaling molecules. Erk was significantly phosphorylated in less than 15 min in all treatment conditions. The phosphorylation state was sustained during the observation period, and the order of intensity was EGF < HGF < HGF + EGF (Fig. 7A). Phosphorylation of Akt gradually increased in all treatment conditions (Fig. 7B). However, the phosphorylation profile of Akt was similar in each treatment condition (Fig. 7E–G), and there was no significant difference in the phosphorylation intensity between treatment conditions (Fig. 7B). In contrast, compared to the untreated cells at 0 h, the HGF-treated cells at 3 h showed a drastic 12-fold increase in the phosphorylation of STAT3 (Fig. 7D), whereas the phosphorylation of Jak2 was only modestly increased after treatment with HGF (Fig. 7C). However, the phosphorylation of Jak1 and Src was not altered by these growth factors (Fig. 7E–G).

3.8. Effects of inhibitors of downstream signaling molecules on HGF- and EGF-induced suppression of CPT-11 cytotoxicity

To further support the involvement of downstream signaling molecules, we conducted inhibitory experiments using U0126, a MEK1/2 inhibitor that decreased the recovered proliferative activity of cells treated with either HGF or EGF alone, whereas the recovered proliferative activity was not altered in cells treated with HGF + EGF (Fig. 8A). In contrast, the recovered proliferative activity induced by treatment with HGF alone, EGF alone, or HGF + EGF was decreased to nearly 40% by A6730, an Akt1/2 inhibitor. Further, simultaneous treatment with both U0126 and A6730 eliminated the recovered proliferative activity induced by EGF alone and by HGF + EGF, but not that induced by HGF alone. While the decrease induced by treatment with U0126 alone or A6730 alone was not complete, the recovered proliferative activity was eliminated by treatment with either WP1066, a Jak2 inhibitor, or cryptotanshinone, a STAT3 inhibitor (Fig. 8B), and the rank order was inversely correlated with the intensity order of STAT3 phosphorylation.

4. Discussion

To the best of our knowledge, this is the first report on HGF- and EGF-induced suppression of the cytotoxicity of CPT-11 and its active metabolite SN-38 in HepG2 cells. This suppression was attributed to the decrease in the conversion of CPT-11 to SN-38 and acceleration of the conversion of SN-38 to SN-38G. Furthermore, we found that the inhibitors of c-Met and EGFR synergistically improve the suppression. These findings suggest that the lifespan of SN-38 in hepatocytes is potently shortened, so that the amount of SN-38 delivered into tumor tissues is decreased in patients with elevated serum levels of HGF and EGF, thereby resulting in the progression of cancer and acquired clinical resistance to CPT-11-based chemotherapy.

CPT-11 has been widely used for treating cancer patients, and its efficacy has been recognized. Regimens using CPT-11 induce excellent tumor regression and drastically lengthen progression-free survival and overall survival, particularly in patients with non-small cell lung cancer and colorectal cancer [25,26]. However, the

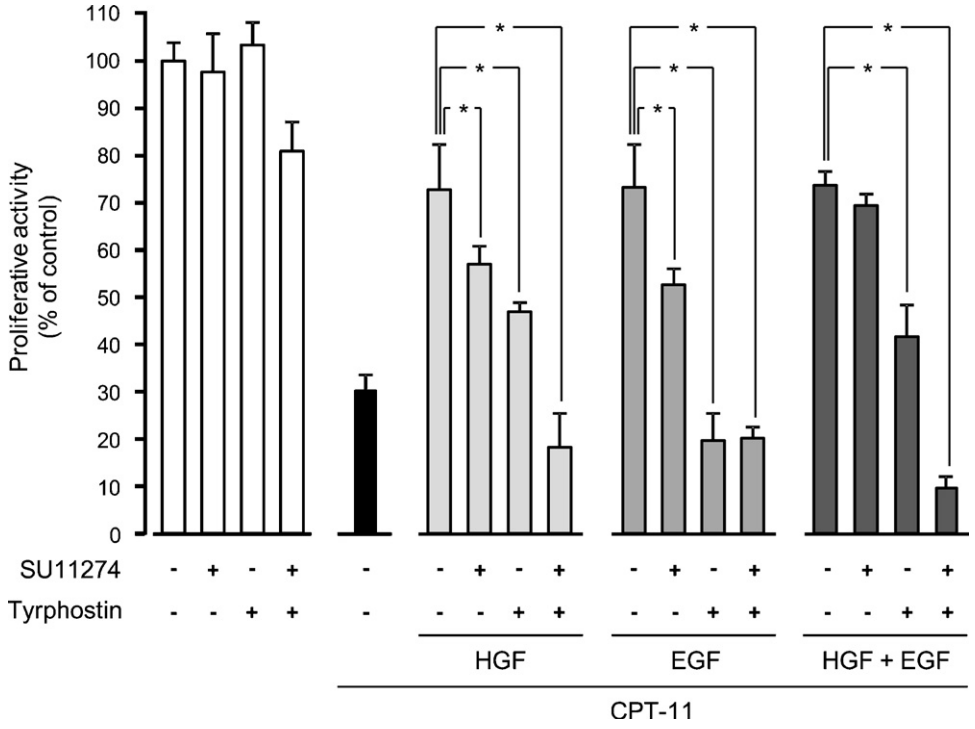

Fig. 5. Inhibitory effects of RTK inhibitors on HGF- and EGF-induced suppression of cytotoxicity of CPT-11. HepG2 cells were cultured for 24 h after treatment with HGF (50 ng/mL), EGF (50 ng/mL), or both. Thereafter, CPT-11 (10.0 μM) was added to the medium, and the cells were further cultured for 96 h. Cells were pretreated with SU11274 (5 μM) and tyrphostin (5 μM) 15 min before administration of growth factors. SU11274 and tyrphostin were used at the concentration at which they produced no effect on the proliferative activity in untreated control cells on their own. The proliferative activity was measured at the end of the incubation period and was expressed as a percentage of that in cells not treated with growth factors. Each bar represents the mean ± SD of 5 experiments. Significant difference between the 2 groups: $^{*}P < 0.01$.

efficacy of CPT-11 is influenced by the amount of SN-38, which is modulated by differences in the expression and activity of CES2 and UGT1A1. In this study, the intracellular AUC of CPT-11, after the addition of CPT-11, was significantly higher in cells treated with HGF + EGF than in untreated cells (Fig. 3B). In contrast, the intracellular AUC of SN-38, after the addition of SN-38, was significantly lower in the HGF- and EGF-treated cells than in the untreated cells (Fig. 3D). These results confirm the effects of HGF and EGF on the expression and/or activity of CES2 and UGT1A1.

However, glucuronidation by UGT1A1 is the only mechanism by which SN-38 is metabolized, whereas CPT-11 is metabolized not only by CES2 but also by CYP3A4 (Fig. 1A). CYP3A4 metabolizes CPT-11 to oxidative metabolites such as 7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin (APC) and 7-ethyl-10-(4-amino-1-piperidino) carbonyloxycamptothecin (NPC) [27,28]. Therefore, we conducted a metabolic experiment using NPA, and we performed Western blotting using the antibody for CYP3A4 to evaluate the involvement of CYP3A4.

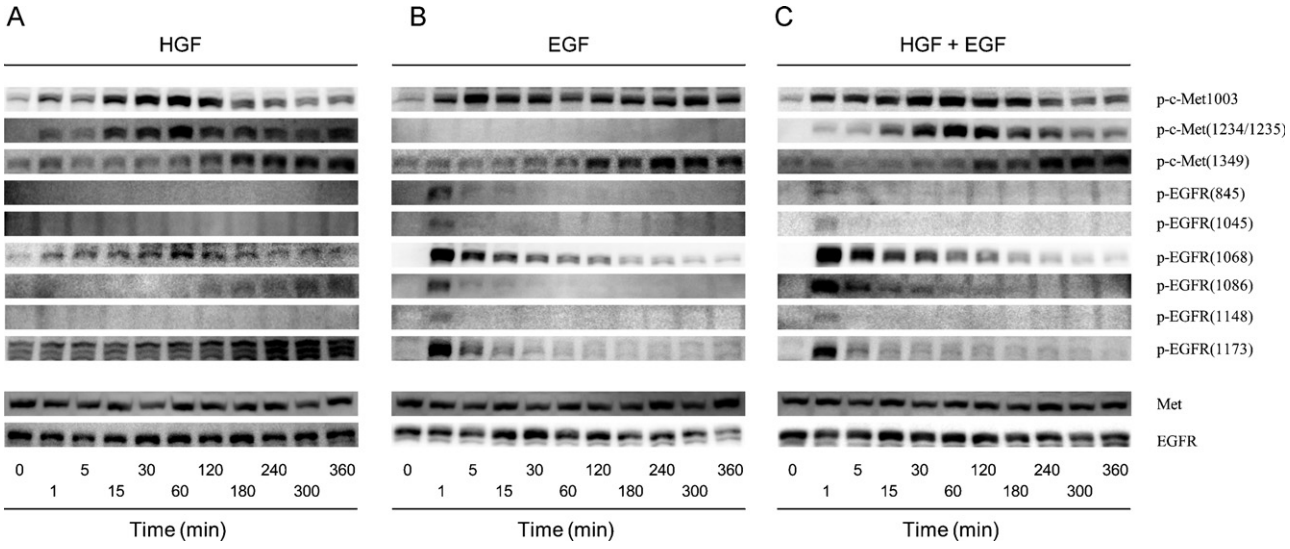

Fig. 6. Transactivation of c-Met and EGFR by EGF and HGF. HepG2 cells were cultured for 24 h in serum-starved medium. Thereafter, HGF (50 ng/mL) (A), EGF (50 ng/mL) (B), or HGF + EGF (C) were added to the medium. Total protein was collected at the indicated times. Western blot analysis was performed in 3 experiments for 3 different preparations, and representative Western blots are shown.

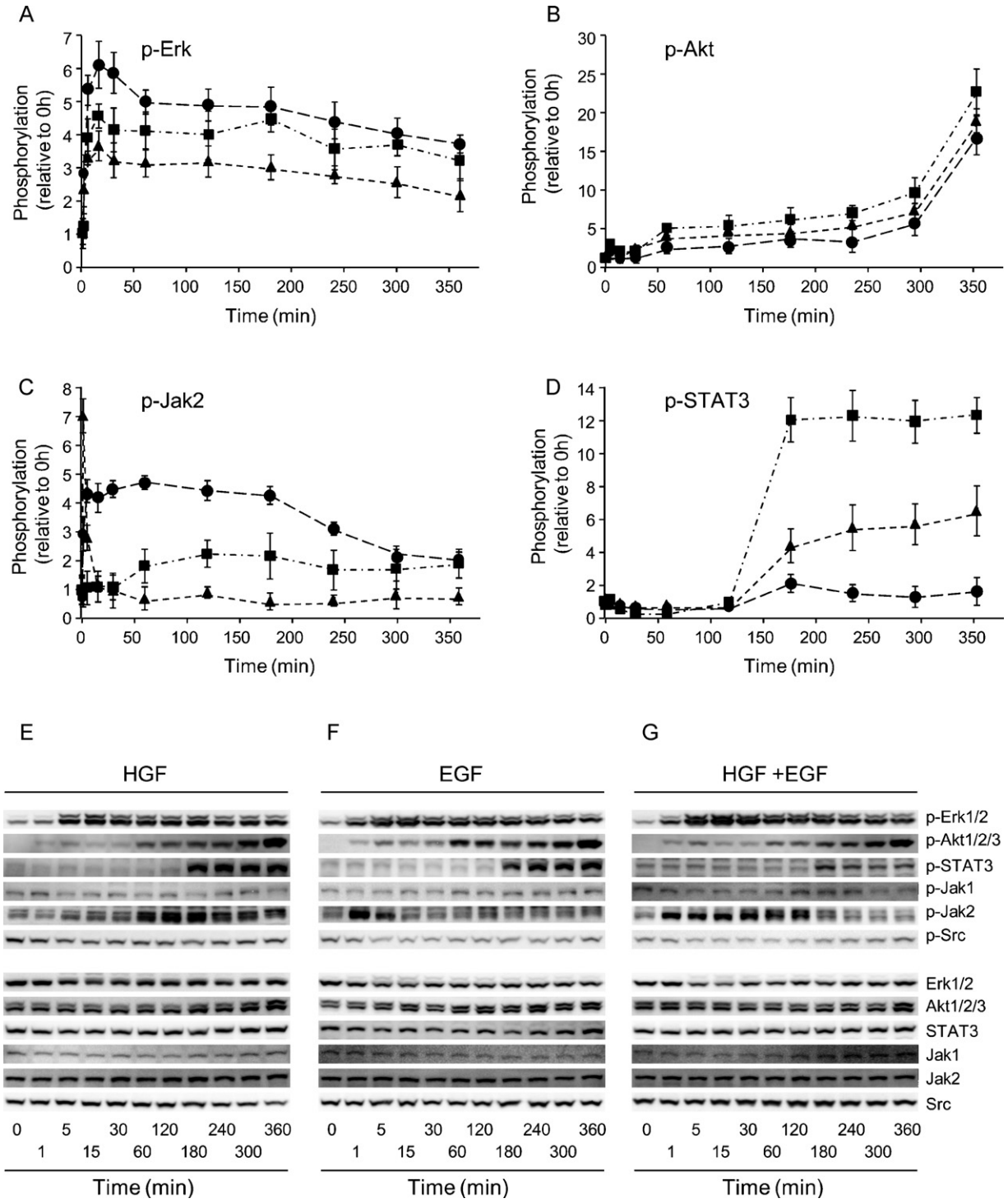

Fig. 7. Time profile for phosphorylation of downstream signaling molecules. HepG2 cells were cultured for 24 h in serum-starved medium. Thereafter, HGF (50 ng/mL), EGF (50 ng/mL), or HGF + EGF were added to the medium. Total protein was collected at the indicated times. The relative amount of phosphorylated protein in each band was quantified by NIH Image-J software, and phosphorylation trends of Erk (A), Akt (B), Jak2 (C), and STAT3 (D) after treatment of growth factors were plotted on graphs. The intensity of phosphorylation is expressed relative to that at 0 min. Western blot analysis was performed in 3 experiments for 3 different preparations, and representative Western blots are shown (E–G). Each bar represents the mean ± SD. Square, HGF; triangle, EGF; circle, HGF + EGF.

Cytosolic proteins harvested from cells treated with HGF + EGF produced significantly lesser amounts of *p*-nitrophenol than those in the untreated cells (Fig. 4A). Further, the expression level of the CYP3A4 protein was slightly decreased by treatment with HGF or EGF (Fig. 4B). These results strongly support the possibility that HGF and EGF have antithetical effects on the expression of CES2 and UGT1A1, i.e., HGF and EGF decrease CES2 and increase UGT1A1.

Nevertheless, although CPT-11 and SN-38, which are lipophilic agents, are taken up by cancer cells via a common carrier by facilitated diffusion, it cannot be completely ruled out that altered expression of influx and efflux transporters is involved in the increase of CPT-11 and the decrease of SN-38 in cells treated with growth factors. We previously reported that epirubicin, a lipophilic antineoplastic agent, is partly taken up by influx transporters [29], and SN-38 is actively excreted outside cells via efflux transporters

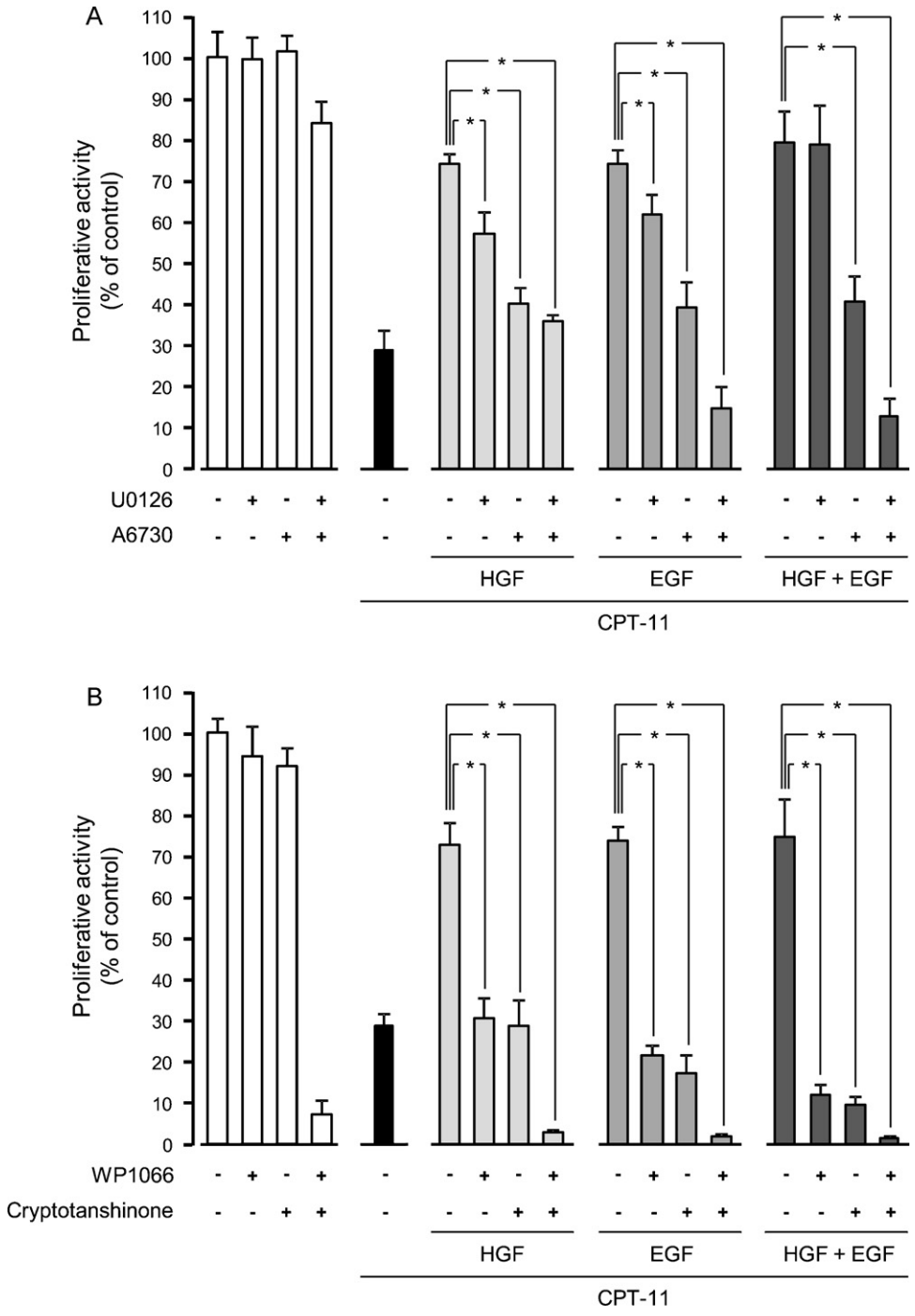

Fig. 8. Comparison of inhibitory effects of inhibitors of downstream signaling molecules on HGF- and EGF-induced proliferative activity recovered from the suppression induced by CPT-11. HepG2 cells were cultured for 24 h after treatment with HGF (50 ng/mL) and EGF (50 ng/mL), which were used alone or in combination. Thereafter, CPT-11 (10.0 μM) was added to the medium, and the cells were further cultured for 96 h. Cells were pretreated with inhibitors 60 min before treatment with growth factors. The inhibitors were used at the concentration at which they produced no effect on the proliferative activity of the untreated control cells on their own. (A) Inhibitory effect of U0126 (2.5 μM), a MEK1/2 inhibitor, and A6730 (5.0 μM), an Akt1/2 inhibitor, or U0126 + A6730. (B) Inhibitory effect of WP1066 (0.5 μM), a Jak2 inhibitor, and cryptotanshinone (0.1 μM), a STAT3 inhibitor, or WP1066 + cryptotanshinone. The proliferative activity was measured at the end of the incubation period and was expressed as a percentage of that in cells not treated with growth factors. Each bar represents the mean ± SD of 5 experiments. Significant difference between 2 groups: $^{*}P < 0.01$.

[30]. Hence, we examined the protein expression levels of the transporters. However, changes in the expression of both influx (Supplementary Fig. S2) and efflux (Supplementary Fig. S3) transporters induced by growth factors were insufficient to alter the intracellular concentration of CPT-11 and SN-38. Therefore, it is plausible that the mechanism by which HGF and EGF suppress CPT-11 cytotoxicity is the antithetical effect of HGF and EGF on the expression of CES2 and UGT1A1.

Since HGF and EGF suppress CPT-11 cytotoxicity, the overexpression of these growth factors and the overactivation of c-Met and EGFR present considerable obstacles to successful chemotherapy using CPT-11. Hence, inhibition of these growth factors in patients with elevated HGF and EGF levels may contribute to overcoming the above-mentioned obstacles. In this study, HGF-induced suppression of CPT-11 cytotoxicity was significantly inhibited by pretreatment with SU11274, a c-Met inhibitor.

Interestingly, although tyrphostin is an EGFR inhibitor, tyrphostin significantly inhibited HGF-induced suppression of CPT-11 cytotoxicity and synergistically potentiated the SU11274-induced inhibition (Fig. 5). The inhibition by tyrphostin indicates that EGFR signaling contributes in part to HGF-induced suppression of CPT-11 cytotoxicity. In fact, HGF induced a significant and consistent increase in EGFR phosphorylation (Fig. 6A). In contrast, EGF-induced suppression of CPT-11 cytotoxicity was partially inhibited by SU11274, but completely inhibited by tyrphostin. However, SU11274 had no effect on the suppression of CPT-11 cytotoxicity in cells simultaneously treated with HGF and EGF. This may be due to negation of the SU11274-induced inhibitory effect by the potentiated phosphorylation of EGFR at the Tyr^{1068} and Tyr^{1086} sites induced by simultaneous treatment with HGF and EGF. Further, although EGF-induced phosphorylation of EGFR was immediate, but transient, HGF-induced phosphorylation of EGFR at the Tyr^{1173} site was gradual, but sustained. However, the intensity of c-Met phosphorylation induced by EGF was similar to that of HGF-induced phosphorylation, except at the $Tyr^{1234/1235}$ site that was not phosphorylated. These results indicate that the EGFR transactivation induced by HGF can be attributed to cytoplasmic mechanisms and that the c-Met transactivation induced by EGF can be attributed to the phosphorylation of kinase domain tyrosines, because of heterodimerization with EGFR.

Downstream signaling molecules such as Erk and Akt are shared by c-Met and EGFR [31]. In this study, the phosphorylation of Erk and Akt consistently increased after the administration of HGF and EGF (Fig. 7A and B). However, although the HGF- and EGF-induced suppression of CPT-11 cytotoxicity was decreased by U0126, an MEK1/2 inhibitor, and A6730, an Akt1/2 inhibitor, the suppression of CPT-11 cytotoxicity in cells simultaneously treated with both HGF and EGF was not decreased by U0126 alone (Fig. 8A). These results may be attributed to the fact that, contrary to the Akt phosphorylation, Erk phosphorylation was potentiated by simultaneous treatment with HGF and EGF. Further, simultaneous treatment with U0126 and A6730 completely inhibited the suppression induced by EGF alone and HGF + EGF, whereas the suppression induced by HGF alone was not completely inhibited. These results indicate that cytoplasmic molecules other than Erk and Akt are more directly involved in the mechanism.

Src and Jak2 have been implicated in transactivation of EGFR [32,33]. However, in this study, Src phosphorylation did not respond to the HGF and EGF treatments. In contrast, Jak2 phosphorylation was increased by HGF and EGF (Fig. 7C). Further, either WP1066, a Jak2 inhibitor, or cryptotanshinone, a STAT3 inhibitor, alone completely inhibited the HGF- and EGF-induced suppression of CPT-11 cytotoxicity (Fig. 8B). Moreover, although simultaneous treatment with HGF and EGF induced Jak2 phosphorylation to a greater degree than that induced by HGF or EGF alone, the degree of STAT3 phosphorylation after simultaneous treatment with HGF and EGF was lesser than that induced by HGF or EGF alone (Fig. 7D). These findings are attributable to the interruption of the signaling transduction from Jak2 to STAT3 in cells simultaneously treated with HGF and EGF. Therefore, the rank order of the inhibitory effects of WP1066 and cryptotanshinone on the suppression of CPT-11 cytotoxicity induced by treatment with growth factors was inversely correlated with the intensity order of STAT3 phosphorylation, but not of Jak2 phosphorylation. Furthermore, the sustained Tyr^{1173} phosphorylation of EGFR did not occur in cells simultaneously treated with HGF and EGF (Fig. 6C). This is also attributed to the above-mentioned interruption of the signaling pathway between Jak2 and STAT3. However, further investigation is needed to elucidate the mechanism.

Reznik et al. reported that HGF transactivates EGFR via EGFR cognate ligands such as transforming growth factor-α and heparin-binding epidermal growth factor-like growth factor [34]. However, these ligands do not appear to be implicated in transactivation of EGFR induced by HGF in HepG2 cells, since more than 6 h are required for the expression of these ligands in the extracellular medium and for EGFR transactivation.

Although HGF stimulation has been reported to promote transactivation of EGFR in multiple cell lines, almost all of these reports have focused on essential HGF-dependent phenotypes such as proliferation, invasion, migration, and cell scatter in cancer cell lines [35–37]. In the present study, by focusing on anticancer drug metabolism, we found that HGF transactivates EGFR via enhanced phosphorylation of downstream signaling molecules in HepG2 cells, thereby resulting in the alteration of expression of metabolic enzymes. However, although STAT3 is considered to be involved in the increase of UGT1A1 expression, further investigation is needed to elucidate the detailed mechanism by which HGF and EGF decrease the expression of CESs.

In conclusion, our findings imply that elevated HGF and EGF or overactivation of c-Met and EGFR result in poor therapeutic effects and clinical resistance in CPT-11-based chemotherapy. Therefore, HGF could be useful as a predictor of clinical resistance in CPT-11-based chemotherapy, and additive treatment with c-Met and/or EGFR inhibitors could be a promising strategy for these patients. However, further clinical investigation is needed to confirm the potential of HGF as a predictor and to verify the benefits of combining CPT-11 with receptor tyrosine kinase inhibitors; such investigations are currently being planned in our institute.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.07.095.

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