

## Effect of c-Met inhibitor SU11274 on hepatocellular carcinoma cell growth

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### Summary

c-Met, a type of receptor tyrosine kinase, may be significantly associated with the progression of hepatocellular carcinoma (HCC). In addition, des- $\gamma$ -carboxyprothrombin (DCP) has been found to interact with c-Met and activate HCC cell growth. Therefore, the functional inhibition of c-Met expressed on HCC cells should arrest HCC cell growth. The present study found that the c-Met inhibitor SU11274 suppressed HCC cell growth by inhibiting the activation of c-Met. Furthermore, this inhibitor also neutralized the activation of HCC cell growth resulting from the addition of DCP. These results suggest that the functional inhibition of c-Met might be a target for the development of chemotherapeutic agents for HCC, and especially those that are positive for expression of DCP.

**Keywords:** c-Met, hepatocellular carcinoma (HCC), inhibitor, growth, des- $\gamma$ -carboxyprothrombin (DCP)

### 1. Introduction

c-Met is a type of receptor tyrosine kinase expressed on the membranes of various cells (1). Hepatocyte growth factor (HGF), also known as scatter factor (SF), has been identified as the ligand for c-Met (2), and their interaction can activate the kinase activity of c-Met to trigger various cellular events, *e.g.* tissue regeneration, cell proliferation, and invasion (3). The phosphorylation of c-Met and subsequent activation of various downstream signal transduction pathways may play a significant role in the progression of hepatocellular carcinoma (HCC) (4-6). Several clinical studies noted overexpression of c-Met in HCC tissue compared to surrounding non-cancerous liver tissue (7-9), and this overexpression was suggested to correlate with

worse behavior of HCC (8). Furthermore, the down-regulation of c-Met expression by transfecting siRNA suppressed the proliferation and invasion of an HCC cell line (10,11). Thus, the expression of c-Met was found to be associated with the progression of HCC and its inhibition may contribute to the development of chemotherapy for HCC. Little research, however, has analyzed the inhibitory effect of c-Met on HCC cells by way of small-molecular compounds.

Des- $\gamma$ -carboxyprothrombin (DCP), also referred to as protein induced by vitamin K absence or antagonist II (PIVKA-II), is known to be an effective tumor marker for HCC (12-14). This tumor marker is regularly used in clinical diagnosis to screen patients with primary HCC and to monitor the recurrence of HCC after curative therapy (15,16). While extensive scientific evidence has been assembled to indicate the clinicopathological significance of DCP, a high level of DCP in the sera of patients with HCC was significantly associated with the presence of vascular invasion followed by the presence of intrahepatic metastasis (16-18). Thus, DCP may serve to predict a worse prognosis of patients with HCC. Additionally, recent molecular biological studies on DCP have

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revealed that DCP activates HCC cell growth (19,20). Gao *et al.* demonstrated that the proliferation of HCC cells increased as a result of DCP in a dose- and time-dependent manner (19). In addition, this action of DCP was also detected *in vivo* analyses using a nude mouse model with HCC cell xenograft (19). A biochemical study performed by Suzuki *et al.* showed that DCP can increase the DNA synthesis of HCC cells *via* the activation of c-Met-Janus kinase (JAK)-signal transducers and activators of the transcription 3 (STAT3) pathway (20). Although previous studies have suggested that the expression and activation of c-Met may play important roles in the growth of HCC cells, and especially those induced by DCP, c-Met inhibitor has never been analyzed in terms of the suppression of HCC cell growth activated by DCP.

The aim of the present study was to clarify the inhibitory effect that a small-molecular inhibitor of c-Met had on HCC cell growth and its relationship to DCP.

## 2. Materials and Methods

### 2.1. Compound

A small-molecular c-Met inhibitor named SU11274 ((3Z)-*N*-(3-chlorophenyl)-3-({3,5-dimethyl-4-[(4-methylpiperazin-1-yl)carbonyl]-1H-pyrrol-2-yl}methylene)-*N*-methyl-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide, Mol. Wt. = 568.09) was purchased from Calbiochem, Inc. (San Diego, CA, USA) (21). This compound was dissolved in dimethyl sulfoxide (DMSO) solution so that the concentration of DMSO was less than 0.1% (v/v) in the analyzed samples.

DCP was generously donated by Eisai Co., Ltd., Tokyo, Japan. DCP was purified from the conditioned media of the DCP-producing cell line PLC/PRF/5 by affinity chromatography with an anti-prothrombin antibody. DCP was distinguished from normal prothrombin using high-performance liquid chromatography (HPLC) (20).

### 2.2. Cell lines

HepG<sub>2</sub> (a well-differentiated HCC cell line) cells were obtained from European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). HuH-7 (a well-differentiated HCC cell line) and HLE (poorly-differentiated HCC cell line) cells were obtained from Health Science Research Resources Bank (HSRRB, Osaka, Japan). These cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C in a humid atmosphere (5% CO<sub>2</sub>-95% air) and were harvested by brief incubation in Enzyme-free Cell Dissociation Solution (Millipore Co., Bedford, MA, USA).

### 2.3. Cell growth assay

Continuously cultured HepG<sub>2</sub>, HuH-7 and HLE cells were harvested in tubes and resuspended in DMEM containing 10% FBS after they were washed with PBS. The cells were seeded in triplicate in 96-well plates at a density of  $6 \times 10^3$  cells in 100  $\mu$ L with or without a concentration of SU11274 and/or DCP and incubated for 24 and 72 h at 37°C in a 5% CO<sub>2</sub> atmosphere. Cell viability was evaluated using a methylthiazole tetrazolium (MTT) cell proliferation assay kit in accordance with the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland). The IC<sub>50</sub> (concentration of drug that inhibits cell growth by 50%) value was calculated using SPSS 11.5 software.

### 2.4. Western blot analysis

Continuously cultured HepG<sub>2</sub>, HuH-7 and HLE cells were harvested in tubes and resuspended in DMEM containing 10% FBS after they were washed with PBS. The cells were seeded in triplicate in 6-well plates at a density of  $3 \times 10^5$  cells in 2 mL with or without a concentration of SU11274 and incubated for 48 h at 37°C in a 5% CO<sub>2</sub> atmosphere. Then, the cells were harvested in tubes and total cell lysates were prepared as previously described (22). Protein concentrations of supernatant were determined using the DC Protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Thirty  $\mu$ g of total cellular proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) transfer membranes by Western blotting. Rabbit anti-human c-Met polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-human phosphorylated-c-Met polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-human phosphorylated-extracellular signal-regulated kinase (ERK) polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA), and mouse anti-human  $\beta$ -actin monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA) were used as primary antibodies.

### 2.5. Statistical analysis

Statistical analysis was performed with the Student's *t*-test using SPSS 11.5 software.  $p < 0.05$  was indicative of a significant difference. All experiments were performed in triplicate.

## 3. Results and Discussion

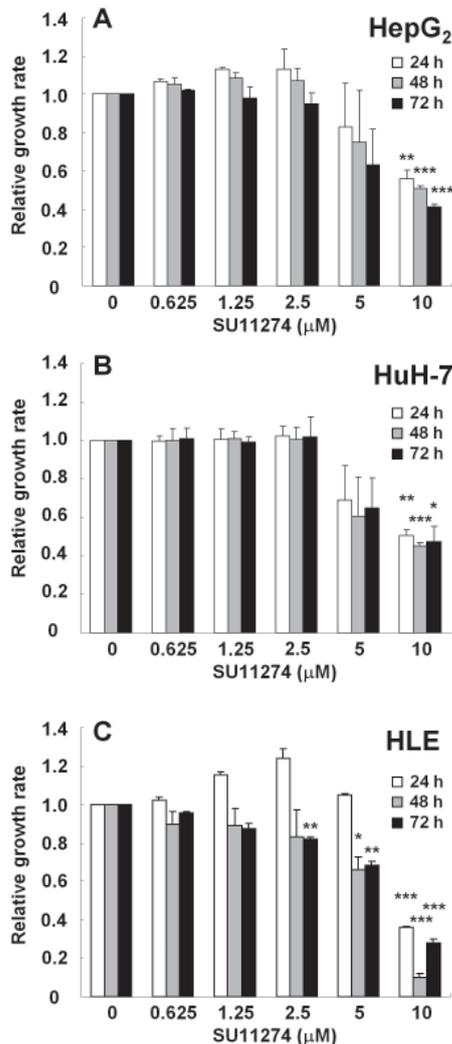
First, the inhibitory effect of c-Met inhibitor SU11274 on HCC cell growth was analyzed. As shown in Figure 1, the growth of all HCC cell lines analyzed was inhibited by the addition of SU11274. This inhibitory

effect was particularly apparent with over 2.5  $\mu\text{M}$  of SU11274. This inhibitory effect occurred in a time- and dose-dependent manner but differed among HCC cell lines with 48 and 72 h of incubation. The growth of HLE cells was gradually inhibited by a lower concentration of SU11274 (0.0625-1.25  $\mu\text{M}$ ) although those concentrations of SU11274 had no significant effect on the growth of HepG<sub>2</sub> and HuH-7. However, similar IC<sub>50</sub> values were noted for the cell lines analyzed; 8.16  $\mu\text{M}$  SU11274 and 48 h of incubation and 6.75  $\mu\text{M}$  SU11274 and 72 h of incubation inhibited HepG<sub>2</sub> cells, 8.24  $\mu\text{M}$  SU11274 and 48 h of incubation and 8.08  $\mu\text{M}$  SU11274 and 72 h of incubation inhibited HuH-7 cells, and 6.36  $\mu\text{M}$  SU11274 and 48 h of incubation and 7.20  $\mu\text{M}$  SU11274 and 72 h of incubation inhibited HLE cells. These results suggest that the addition of the small-molecular c-Met inhibitor SU11274 may inhibit the growth of HCC cells.

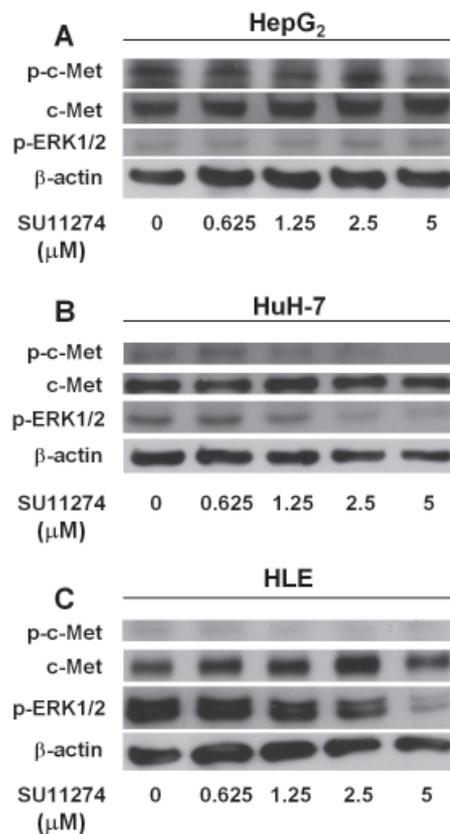
Next, Western blot analysis was performed to clarify

the changes in c-Met expression or its activation profile as a result of SU11274. All HCC cell lines analyzed were positive for c-Met expression, and this level of expression in those HCC cell lines did not change significantly with the addition of SU11274 (Figure 2). In contrast, the expression of phosphorylated-c-Met gradually decreased in a dose-dependent manner with SU11274. The rate of inhibited expression of phosphorylated-c-Met in the presence of 5  $\mu\text{M}$  SU11274 was 43%, 84%, and 53% for HepG<sub>2</sub>, HuH-7, and HLE cells, respectively. In addition, the expression of phosphorylated-ERK, the downstream kinase of the signal transduction pathway activated by c-Met, also decreased in a dose-dependent manner with SU11274. The rate of inhibited expression of phosphorylated-ERK in the presence of 5  $\mu\text{M}$  SU11274 was 18%, 39%, and 78% for HepG<sub>2</sub>, HuH-7, and HLE cells, respectively. These results suggest that the down-regulation of c-Met activation by SU11274 may inhibit the growth of HCC cells.

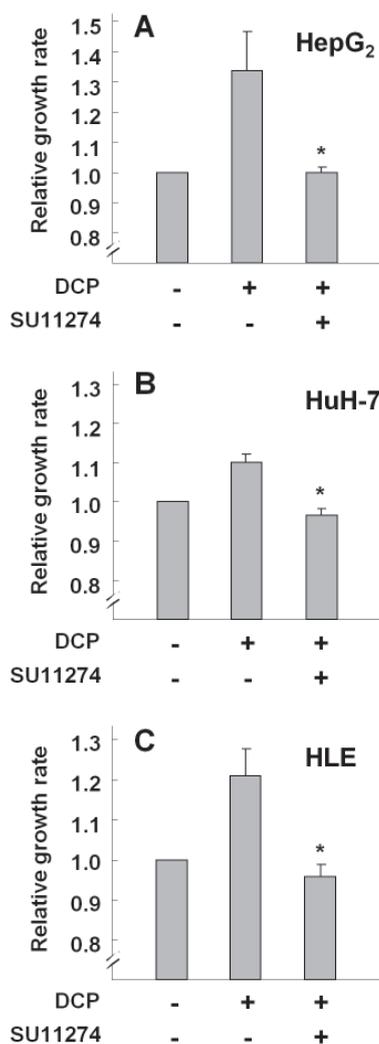
As described previously, c-Met may act as a target receptor of DCP and trigger the activation of HCC cell growth (20). Therefore, the inhibition of c-Met presumably suppresses HCC cell growth induced by DCP. The present study investigated the ability of SU11274 to inhibit DCP by simultaneously



**Figure 1. Relative growth rates of (A) HepG<sub>2</sub>, (B) HuH-7 and (C) HLE cells in the various concentrations of SU11274.** White column, 24 h; gray column, 48 h; solid column, 72 h incubation with SU11274. The data represent mean  $\pm$  S.D. \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs. control with 0  $\mu\text{M}$  SU11274.



**Figure 2. Expressed levels of phosphorylated-c-Met, overall c-Met, and phosphorylated-ERK in (A) HepG<sub>2</sub>, (B) HuH-7 and (C) HLE cells detected by Western blot analysis.**



**Figure 3. Relative growth rates of (A) HepG<sub>2</sub>, (B) HuH-7 and (C) HLE cells in the presence of DCP (160 ng/mL) and/or SU11274 (2.5  $\mu$ M).** Whereas the growth of HCC cells was activated by the addition of DCP, the presence of SU11274 with DCP significantly inhibited this action. The data represent mean  $\pm$  S.D. ( $n = 3$ ). \*  $p < 0.05$  vs. sample with DCP.

incubating HCC cells with these substances for 48 h. The concentration of DCP was set at 160 ng/mL in accordance with the results of a previous study (19). In addition, the concentration of SU11274 was set at 2.5  $\mu$ M because the inhibitory effect on cell growth was detected with 5  $\mu$ M and 48 h of incubation (Figure 1). As a result, the incubation of HCC cells with DCP activated HCC cell growth, as shown in Figure 3. Previous studies also analyzed the effect of DCP on HCC cell growth using HepG<sub>2</sub> (19) and results of those studies coincided with the results of the present study. The results indicated that the relative extent of growth in the presence of DCP differed among the HCC cell lines analyzed, and this difference may be due to their sensitivity to DCP. The effect of SU11274 on DCP was also analyzed. The activation of HCC cell growth by DCP was neutralized by the simultaneous addition of SU11274 (Figure 3). These results indicate that the

growth of HCC cells was up-regulated by DCP via the activation of c-Met and that these biological events can be inhibited by c-Met inhibitor.

This study was performed with the aim of evaluating the effectiveness of the c-Met inhibitor SU11274 as an anti-cancer chemotherapeutic agent in treating HCC cells. Results revealed that SU11274 had the ability to inhibit HCC cell growth. Several mechanisms whereby the activation of c-Met leads to HCC progression have been suggested. These mechanisms involve ERK-mediated and phosphatidylinositol 3 kinase (PI3K)-mediated pathways (23). As described previously, the JAK-STAT3 pathway may also play an important role in the up-regulation of HCC cell proliferation induced by DCP (20). Thus, various pathways triggered by activated c-Met might regulate the behavior of HCC. Further clarification of the c-Met-mediated mechanism of HCC progression may lead to the development of novel chemotherapeutic agents for HCC.

In conclusion, this study reported that inhibition of c-Met by the small-molecular inhibitor SU11274 arrested HCC cell growth. In addition, DCP's action as a growth factor might be neutralized by the inhibition of c-Met activation. Various researchers are developing novel c-Met inhibitors (24,25), and these inhibitors may prove of use in chemotherapy to treat HCC.

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