Antitumor activities of JTP-74057 (GSK1120212), a novel MEK1/2 inhibitor, on colorectal cancer cell lines in vitro and in vivo

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Abstract. The MAPK pathway is one of the most important pathways for novel anticancer drug development. We performed high-throughput screening for compounds that induce expression of p15INK4a, and identified JTP-74057 (GSK1120212), which is being evaluated in ongoing phase I, II and III clinical trials. We characterized its antitumor activities in vitro and in vivo. JTP-74057 strongly inhibited MEK1/2 kinase activities, but did not inhibit another 98 kinase activities. Treatment by JTP-74057 resulted in growth inhibition accompanied with upregulation of p15INK4a and/or p27KIP1 in most of the colorectal cancer cell lines tested. Daily oral administration of JTP-74057 for 14 days suppressed tumor growth of HT-29 and COLO205 xenografts in nude mice. Notably, tumor regression was observed only in COLO205 xenografts, and COLO205 was much more sensitive to JTP-74057-induced apoptosis than HT-29 in vitro. Treatment with an Akt inhibitor enhanced the JTP-74057-induced apoptosis in HT-29 cells. Finally, JTP-74057 exhibited an additive or a synergistic effect in combination with the standard-of-care agents, 5-fluorouracil, oxaliplatin or SN-38. JTP-74057, a highly specific and potent MEK1/2 inhibitor, exerts favorable antitumor activities in vitro and in vivo. Sensitivity to JTP-74057-induced apoptosis may be an important factor for the estimation of in vivo efficacy, and sensitivity was enhanced by an Akt inhibitor. These results suggest the usefulness of JTP-74057 in therapeutic applications for colorectal cancer patients.

Introduction

The MAPK pathway is a central signal transduction pathway of cell proliferation and survival (1,2). Various growth factors bind to their receptor tyrosine kinases on the cell surface and induce dimerization and autophosphorylation of the receptors followed by association of adaptor proteins. Subsequently, the guanine nucleotide exchange factor Sos activates Ras by catalyzing the replacement of GDP with GTP. GTP-bound Ras then recruits the serine/threonine kinase Raf (A-Raf, B-Raf or c-Raf) to the plasma membrane where it becomes activated, resulting in phosphorylation of MEK1/2 by the Raf kinase. Activated MEK1/2 in turn phosphorylate ERK1/2 at specific threonine and tyrosine residues. The phosphorylated ERK1/2 phosphorylate and activate a variety of substrates, such as p90RSK1. Also, ERK1/2 themselves can translocate to the nucleus and induce transcription of genes for cell proliferation and survival. In human malignant tumors, the Raf-MEK-ERK pathway is often hyperactivated through aberrant activation of the receptor tyrosine kinases or gain-of-function mutations in the Ras or B-Raf gene (1). One of the Ras gene family proteins, K-Ras, is reported to be mutated in 90% of pancreatic cancers, 45% of colorectal cancers and 35% of non-small cell lung cancers (3,4). B-Raf mutations have been identified in 66% of melanomas, 12% of colorectal cancers and 35-70% of papillary thyroid cancers (5-9). The overexpression and/or mutation of epidermal growth factor (EGF) receptor (EGFR), erbB2, platelet-derived growth factor receptor and other growth factor receptors have also been observed in many types of cancers (10,11). Thus, a component of the Raf-MEK-ERK pathway has been considered as an attractive target for a novel approach to chemotherapy. CI-1040 (also called PD184352) was the first MEK1/2 inhibitor evaluated in clinical trials (12,13), although its clinical development was discontinued due to insufficient efficacy. The second generation MEK1/2 inhibitors, PD0325901 and AZD6244 (ARRY-142886), which have shown better efficacy than CI-1040 in preclinical models, have also been tested in clinical trials (1,2,14,15).

Based on our original screening strategy for molecular-targeting agents, we initiated a high-throughput screening for compounds that induce expression of cyclin-dependent kinase (CDK) inhibitors, such as p15INK4a, p21WAF1 or p27KIP1. Notably,
we and others found that several molecular-targeting agents  
go against cancer, such as HDAC inhibitors, gefitinib, imatinib, or  
trastuzumab enhanced expression of CDK inhibitors (16-19), 
and we identified several novel molecular-targeting agents for  
clinical use by the screening vice versa (20), suggesting that  
our screening methods are useful for the development of novel  
molecular-targeting drugs for cancer. Based on this strategy, we  
conducted a high-throughput screening for compounds that  
induce the expression of p15\^{INK4b}, an endogenous inhibitory  
protein of CDK4/6, and found a pyridopyrimidine derivative  
JTP-70902 (21). Subsequent analysis revealed that JTP-70902  
is a novel MEK1/2 inhibitor, which has comparable efficacy  
to AZD6244 (14,21). Through the further efforts of medicinal  
chemistry, we discovered JTP-74057 [its dimethyl sulfoxide  
(DMSO)-solvent form is also called GSK1120212], which  
has the highest MEK1/2 inhibitory activity within the series.  
JTP-74057 showed much more potent antitumor activity than  
the second generation MEK inhibitors, such as PD0325901 or  
AZD6244, in vivo and is being evaluated in phase III clinical  
trial in melanoma patients with B-Raf mutations. Here, we  
report the biological profile of JTP-74057 and discuss its anti-  
tumor effects on human colorectal cancer cell lines in vitro  
and in vivo.

Materials and methods

Chemicals and reagents. JTP-74057, N-(3-[3-cyclopropyl-  
5-(2-Fluoro-4-Iodophenylamino)-6,8-dimethyl-2,4,7-trioxo-  
3,4,6,7-tetrahydro-2H-pyrido[4,3-d]pyrimidin-1-yl]phenyl)  
acetamide and SN-38 were synthesized at Japan Tobacco  
Inc. (Osaka, Japan). API-2 was purchased from Calbiochem  
(Darmstadt, Germany). 5-Fluorouracil (5-FU) and oxaliplatin  
were purchased from Wako Pure Chemical Industries (Osaka,  
Japan) or Enzo Life Sciences (Farmington, NY), respectively.  
A vehicle of 0.1% DMSO was used for test compounds  
in vitro. Antibodies against p15\textsuperscript{INK4\textsubscript{b}}, p27\textsuperscript{KIP1} and  
\(\alpha\)-tubulin were purchased from Santa Cruz Biotechnology  
(Darmstadt, Germany). Antibodies for ERK1/2, phospho-ERK1/2  
and the apoptosis sampler kit, which contains antibodies for the  
cleaved/non-cleaved form of caspase-3/7/9 and PARP, were from  
Cell Signaling Technology (Danvers, MA). HRP-labeled secondary  
antibodies and the ECL Western blot detection reagent were  
purchased from GE Healthcare Bio-Science (Piscataway, NJ).  
Recombinant kinases of MEK1 (inactive), MEK2 (inactive),  
ERK2 (inactive), c-Raf (truncated form, active) and B-Raf  
(V599E, active) were obtained from Millipore (Billerica, MA).

Protein kinase assays. A Raf-MEK-ERK cascade kinase assay  
was carried out as previously described (21). Briefly,  
non-phosphorylated myelin basic protein, (MBP) (Millipore)  
was coated onto an ELISA plate, and the active form of B-Raf/c-Raf  
was mixed with unphosphorylated MEK1/MEK2 and ERK2  
in 10 µM ATP and 12.5 mM MgCl\textsubscript{2} containing MOPS buffer  
in the presence of various concentrations of JTP-74057. The  
phosphorylation of MBP was detected by the anti-phospho-  
MBP antibody. Kinase inhibitory activities against a total of 99  
kinesases were tested by Millipore's kinase profiler at 10 µM ATP.

Cell cultures and growth inhibition assays. Human colorectal  
cancer cell lines, HT-29, HCT-15, HCT116, COLO205, LS-174T,  
SW480, SW620 and T84, were obtained from American Type  
Culture Collection (Manassas, VA). LoVo and COLO320 DM  
drew from Health Science Research Resources Bank (Osaka,  
Japan). These cells were maintained in media recommended  
by the providers. Exponentially growing cells were preincubated  
in 96-well tissue culture plates for 24 h and then exposed  
to JTP-74057. Cell growth was determined by an \textit{in vitro}  
toxicity assay kit, sulfonamide B based (Sigma-Aldrich,  
St. Louis, MO). For combination studies, two compounds  
were simultaneously added to the HT-29 cells and incubated  
for 72 h. In the presence of various concentrations of compound A,  
the 50% inhibitory concentration (IC\textsubscript{50}) values of compound B  
determined. Then, the fixed concentration of compound A  
versus the IC\textsubscript{50} value of compound B was plotted. Conversely, the  
IC\textsubscript{50} values of compound A were determined in the presence  
of various concentrations of compound B and plotted.

Western blot analysis. Western blot analysis of the cell lysates  
was carried out according to a standard method. Peroxidase-  
based chemiluminescence was developed by addition of  
ECL, and the bands were detected using an LAS 3000 image  
 analyzer (Fuji Film, Tokyo, Japan).

Apoptosis assay. Cells were treated with various concentrations  
of JTP-74057 in 100-mm dishes for 3 or 4 days. Both floating  
and adherent cells were collected and fixed with 70% ethanol.  
After washing with PBS, the cells were suspended in 100 µl/ml  
RNase and 25 µl/ml propidium iodide (PI) and incubated at 37˚C  
for 30 min in the dark. The DNA content of each single cell was  
determined using the flow cytometer Cytomics FC500 (Beckman  
Coulter, Brea, CA) or Guava EasyCyte plus (Millipore).

Nude mouse xenograft model. Female BALB/c-nu/nu mice  
obtained from Charles River Japan (Astugi, Japan) were used.  
On day 0, HT-29 cells or COLO205 cells suspended in ice-cold  
HBSS (-) were inoculated subcutaneously into the right flank  
of the mice at 5x10\textsuperscript{6} cells/100 µl/site or 1x10\textsuperscript{6} cells/100 µl/site,  
respectively. The acetic acid-solvated form of JTP-74057 was  
dissolved in 10% Cremophor EL-10% PEG400 and was administered  
intraperitoneally once daily for 14 days from the day when the  
mean tumor volume reached 100 mm\textsuperscript{3}. The tumor length [L (mm)]  
and width [W (mm)] were measured using a microgauge twice a  
week after commencement of dosing, and the tumor volume was  
calculated using the following formula: tumor volume (mm\textsuperscript{3})  
= L \times W \times W/2. All procedures relating to the use of animals  
in this study were reviewed and approved by the Institutional  
Animal Care and Use Committee of Japan Tobacco.

Statistical analysis. The data were expressed as means ± SD  
from the indicated number of samples. Multiple comparisons  
were assessed by one-way ANOVA followed by Dunnett's test  
(Statlight, YUKMS, Tokyo, Japan) and two-sample comparis  
on were assessed by the Student's t-test. Differences were  
considered as significant at p<0.05.

Results

\textit{JTP-74057 is a potent and highly specific MEK1/2 inhibitor.}  
The inhibitory activity of JTP-74057 against MEK1/2 was  
tested using the Raf-MEK-ERK cascade kinase assay using  

recombinant proteins. JTP-74057 inhibited the phosphorylation of MBP regardless of the isotype of Raf and MEK, with IC₅₀ values ranging from 0.92 to 3.4 nM (Table I), which indicates JTP-74057 has 3- to 30-fold stronger activity than that of its parent compound JTP-70902 (21). JTP-74057 demonstrated no inhibition of the kinase activities of c-Raf, B-Raf, ERK1 and ERK2 (Table IB and data not shown), suggesting that JTP-74057 inhibits the Raf-MEK-ERK cascade reaction by inhibiting MEK1/2 activity. In addition, JTP-74057 did not show drastic inhibitory activity against the other 98 kinases examined (Table IB). The data are consistent with the finding that JTP-74057 directly binds to MEK1/2 by compound-immobilized affinity chromatography (data not shown). Together, these results indicate that JTP-74057 is a highly specific MEK1/2 inhibitor.

**Anti-proliferative effect of JTP-74057 on 10 human colorectal cancer cell lines.** Next, we examined the anti-proliferative effects of JTP-74057 using 10 human colorectal cancer cell lines. JTP-74057 inhibited the growth of 9 out of 10 cell lines (Table II). HT-29 and COLO205 cells, which are known to have a constitutively active B-Raf mutant, were most sensitive to JTP-74057, showing subnanomolar IC₅₀ values. The cell lines bearing a K-Ras mutation showed a wide range of sensitivity to JTP-74057. In contrast, COLO320 DM cells, bearing the wild-type gene in both B-Raf and K-Ras, were found to be resistant to JTP-74057 even at 10,000 nM. Similar results were reported regarding the other MEK inhibitors, PD0325901 and AZD6244 (15,22). All sensitive cell lines showed cell-cycle arrest at the G₁ phase after treatment with JTP-74057 for 24 h (data not shown). As we reported previously, MEK inhibitors induced the expression of CDK inhibitors p15INK4b and p27KIP1, and we examined the expression level of p15INK4b and p27KIP1 in these cell lines after treatment with JTP-74057. As shown in Fig. 1, both p15INK4b and p27KIP1 were upregulated in the HT-29, COLO205, HCT-15, HCT116 and LoVo cells, whereas either p15INK4b or p27KIP1 was upregulated in the LS-174T, SW480, SW620 and T84 cells. Most importantly, the resistant cell line, COLO320 DM, showed no induction of p15INK4b or p27KIP1 by JTP-74057, suggesting that upregulation of either p15INK4b or
Table I. Continued.

B. Effect of JTP-74057 on various kinase activities (JTP-74057, 10 μM).

<table>
<thead>
<tr>
<th>Kinase</th>
<th>% of control</th>
<th>Kinase</th>
<th>% of control</th>
<th>Kinase</th>
<th>% of control</th>
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<tbody>
<tr>
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<td>Abl (T315I)</td>
<td>95</td>
<td>PKCα</td>
<td>101</td>
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<tr>
<td>ALK</td>
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<td>Arg</td>
<td>111</td>
<td>PKCβ</td>
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<tr>
<td>Aurora-A</td>
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<td>Btk</td>
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<tr>
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<td>Btk</td>
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<td>101</td>
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<tr>
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<td>PKCζ</td>
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<tr>
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<td>CDK6/cyclin D3</td>
<td>107</td>
<td>PKCθ</td>
<td>114</td>
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<tr>
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<td>CDK7/cyclin H/MAT1</td>
<td>106</td>
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<td>109</td>
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<tr>
<td>EphB2</td>
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<td>124</td>
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<td>102</td>
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<tr>
<td>EphB4</td>
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<tr>
<td>FGFR3</td>
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<tr>
<td>Fms</td>
<td>128</td>
<td>Fms</td>
<td>107</td>
<td>PKCε</td>
<td>102</td>
</tr>
</tbody>
</table>

Abl, Abl tyrosine kinase; T315I, T315I mutations of Thr-315 to Ile; ALK, anaplastic lymphoma kinase; Arg, Abl-related gene product tyrosine kinase; Aurora-A, Aurora-A kinase; Ax1, Ax1 receptor tyrosine kinase; Bmx, BMX non-receptor tyrosine kinase; BTK, bruton agammaglobulinemia tyrosine kinase; CaMK, calcium/calmodulin-dependent protein kinase; CDK, cyclin-dependent kinase; MAT, menage a trois; CHK, checkpoint kinase; CK, casein kinase; c-RAF, Raf proto-oncogene serine/threonine protein kinase; CSK, C-terminal Src kinase; cSRC, SRC proto-oncogene tyrosine protein kinase; EGFR, epidermal growth factor receptor kinase; EphA2, Ephrin-A2 receptor tyrosine kinase protein kinase; EphB2, Ephrin-B2 receptor tyrosine kinase protein kinase; EphB4, Ephrin-B4 receptor tyrosine kinase protein kinase; ErbB4, ErbB4 tyrosine kinase; Fes, Fes tyrosine protein kinase; FGFR, fibroblast growth factor receptor kinase; Fgr, Fgr tyrosine protein kinase; Flt, Fms-like tyrosine protein kinase; Fms, Fms tyrosine protein kinase; Fyn, Fyn tyrosine protein kinase; GSK, glycogen synthase kinase; IκB, IκB protein kinase; IκB kinase; IR, insulin receptor; JNK, c-Jun N-terminal kinase; Lck, lymphocyte-specific protein-tyrosine kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; Mek, MAPK/ERK kinase; Met, c-Met receptor tyrosine kinase; MKK, MAPK kinase; MST, mammalian homologue STE20-like kinase; NEK, non-inherited maternal MHC antigens (NIMA)-related kinase; p70S6K, 3'-phosphoinositide-dependent kinase; PKA, cAMP-dependent protein kinase; PKB, protein kinase B; PKC, protein kinase C; PKD, protein kinase D; Plk3, polo-like kinase-3; PRAK, p38-related/activated protein kinase; PRK, protein kinase C-related kinase; Ret, ret receptor tyrosine kinase; ROCK-II, Rho-associated coiled-coil-containing protein kinase 2; Ros, Ros receptor protein tyrosine kinase; Rse, Rse receptor-type tyrosine kinase; Rsk, p90 ribosomal S6 kinase; SAPK, stress-activated protein kinase; SGK, serum/glucocorticoid-inducible kinase; Syk, spleen tyrosine kinase; Tie2, Tie-2 receptor tyrosine kinase; Trk, tyrosine kinase; Yes, Yes kinase; ZAP-70, ζ-chain (TCR)-associated protein kinase 70 kDa.
p27KiP1 is a prerequisite for growth inhibition of MEK inhibitors.

We also tested the phosphorylation level of ERK1/2 by Western blotting. Constitutive ERK phosphorylation was detected in all the cell lines except for COLO320 DM, and it was inhibited by JTP-74057 (Fig. 1). These results suggest that growth inhibitory activity of JTP-74057 is strongly dependent on the MEK-ERK pathway in tumor cells.

Table II. In vitro growth inhibitory activities of JTP-74057 against human colorectal cancer cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Ras/B-Raf mutation</th>
<th>IC50 value (nM)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-29</td>
<td>B-Raf</td>
<td>0.48</td>
</tr>
<tr>
<td>COLO205</td>
<td>B-Raf</td>
<td>0.52</td>
</tr>
<tr>
<td>COLO320 DM</td>
<td>WT</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>HCT-15</td>
<td>K-Ras</td>
<td>174</td>
</tr>
<tr>
<td>HCT 116</td>
<td>K-Ras</td>
<td>5.7</td>
</tr>
<tr>
<td>LS-174T</td>
<td>K-Ras</td>
<td>4.1</td>
</tr>
<tr>
<td>SW480</td>
<td>K-Ras</td>
<td>8.5</td>
</tr>
<tr>
<td>SW620</td>
<td>K-Ras</td>
<td>2.3</td>
</tr>
<tr>
<td>T84</td>
<td>K-Ras</td>
<td>36</td>
</tr>
<tr>
<td>LoVo</td>
<td>K-Ras</td>
<td>2.2</td>
</tr>
</tbody>
</table>

\(^a\)Mean of 3 experiments. Various human colorectal cancer cell lines were treated with various concentrations of JTP-74057 for 72 h. Viable cell number was determined by Sulforhodamine B assay.

p27KiP1 is a prerequisite for growth inhibition of MEK inhibitors.

Oral dosing of JTP-74057 resulted in tumor growth inhibition in a nude mouse xenograft model. JTP-74057 was evaluated in mice for antitumor activity against HT-29 xenografts. HT-29 cells were inoculated into nude mice, and JTP-74057 was orally administered once daily for 14 days. Both 0.3 mg/kg and 1 mg/kg of JTP-74057 were effective in inhibiting the HT-29 xenograft growth, and 1 mg/kg of JTP-74057 blocked the tumor increase almost completely (Fig. 2A). Neither significant changes in body weight nor gross lesions at autopsy were observed in the mice treated with 1 mg/kg of JTP-74057. We
also found that the phosphorylation of ERK1/2 was completely inhibited in the established tumor tissues by single oral dose of 1 mg/kg JTP-74057 (Fig. 2B), and both p15\textsuperscript{INK4b} and p27\textsuperscript{KIP1} protein levels were upregulated after 14 days of treatment with JTP-74057 (Fig. 2C). These results indicate that JTP-74057 exerts an antitumor effect \textit{in vivo} by the mechanisms of action that were demonstrated in the \textit{in vitro} studies. The antitumor activity of JTP-74057 was also evaluated in the COLO205 xenograft model. As shown in Fig. 2D, tumor regression was observed even at a dose of 0.3 mg/kg. At a dose of 1 mg/kg, a complete regression was obtained in 4 out of 6 mice in which the tumor degenerated to the point that tumor volume was not measurable. Again, neither significant changes in body weight nor gross lesions at autopsy were observed in the mice treated with JTP-74057.

\textbf{Apoptosis induction by JTP-74057 in HT-29 and COLO205 cells \textit{in vitro}.} Despite the fact that JTP-74057 inhibited the proliferation of HT-29 and COLO205 cells \textit{in vitro}, the COLO205 tumors were much more sensitive to JTP-74057 than HT-29 tumors \textit{in vivo}. To address this issue, apoptosis induction by JTP-74057 was analyzed \textit{in vitro}. Both cell lines were treated with JTP-74057 for 4 days. On days 1 and 4, cells were collected and the intracellular DNA was measured by flow cytometry. As shown in Fig. 3A, on day 1, the G1 peak was elevated compared to that on day 0 at all
combination treatment of 10 nM of JTP-74057, which did not induce apoptosis, and Akt inhibitor, API-2, in HT-29 cells. As shown in Fig. 3C, API-2 significantly enhanced apoptosis in HT-29 cells treated with JTP-74057 as expected.

**Combination effect of JTP-74057 and standard-of-care agents on the growth inhibition in HT-29 cells.** Finally, the combination effects of JTP-74057 with standard-of-care agents for colorectal cancer, 5-FU, oxaliplatin and SN-38 (the active metabolite of irinotecan) were investigated *in vitro* using the isobologram method as described in Materials and methods. JTP-74057 showed an additive effect in combination with 5-FU or oxaliplatin, and a synergistic effect in combination with SN-38 (Fig. 4A-C). The IC_{50} values of SN-38 added with oxaliplatin were plotted below the line connecting the IC_{50} values in mono-treatment, showing synergism (Fig. 4D). The data are consistent with a previous report (26). Likewise, the effect of SN-38 treatment in combination with vincristine demonstrated antagonism in terms of the isobologram (Fig. 4E), consistent with a previous report (27).

**Discussion**

The MEK-ERK pathway regulates the expression of various cell cycle-related proteins. MEK inhibitors induce CDK inhibitors (CKIs), p15^{INK4b} and p27^{KIP1}, and downregulate cyclin D1, cyclin E and cyclin A, which in turn leads to reactivation of retinoblastoma gene (RB) protein and repression of a number of cell cycle-promoting molecules, so that finally the cell cycle is arrested at the G_{1} phase (15,21,28-30). In the present study, we utilized a panel of 10 human colorectal cancer cell lines and investigated the relationship between CKI induction and growth inhibition by the MEK inhibitor, JTP-74057. JTP-74057 inhibited cell proliferation of all the cell lines except for COLO320 DM cells, and either p15^{INK4b} or p27^{KIP1}, or both, were upregulated after the treatment in these sensitive cell lines. On the other hand, neither p15^{INK4b} nor p27^{KIP1} was induced by JTP-74057 in COLO320 DM cells. In contrast, we previously demonstrated that p15^{INK4b(-/-)} mouse embryonic fibroblasts (MEFs) were more resistant to the growth-inhibitory effect of the MEK inhibitor JTP-70902 than wild-type MEFs (21). Taken together, these results suggest that p15^{INK4b} and p27^{KIP1} at least partially play roles in cell cycle arrest by a MEK inhibitor.

However, the relative importance of these events in growth arrest may vary in each cell line. For instance, there are two conflicting reports regarding the necessity of p27^{KIP1} induction in the cell cycle arrest by MEK inhibitor U0126. It was indispensable in pancreatic cell lines (29), but not in colorectal cancer cell lines (30). We therefore hypothesized that other factors such as induction of apoptosis may be involved in the growth suppression by MEK inhibitors.

We next performed an *in vivo* study and found that JTP-74057 exerted excellent efficacy both in HT-29 and COLO205 xenograft models. As shown in Fig. 2B, COLO205 tumors were almost completely diminished by daily oral administration with JTP-74057 at a dose of 1 mg/kg/day. This efficacy was superior to those of the second generation MEK inhibitors, as PD0325901 or AZD6244 induced the tumor regression at 25 or 50 mg/kg/day in the COLO205
xenograft model, respectively (15,22). Tumor regression by JTP-74057 was observed in the COLO205 tumors, but not in the HT-29 tumors, despite comparable IC_{50} values observed in the in vitro assay. As there was no substantial difference in the engraftment rates or the doubling time between the two cell lines, it is not likely that host defense against these cell lines is different. By further analysis in vitro, we found that the COLO205 cells were much more sensitive to JTP-74057-induced apoptosis than the HT-29 cells. This may be one of the major reasons why JTP-74057 achieved tumor regression only in the COLO205 xenograft model.

Many proteins involved in apoptosis are regulated by the MEK-ERK pathway (2). Indeed, MEK inhibition by JTP-74057 resulted in caspase-dependent apoptosis. Many types of cancer cells acquire resistance to apoptosis by activating several signal transduction pathways such as the nuclear factor (NF)-κB pathway, the MEK-ERK pathway or the PI3K-Akt pathway. Mutation of PI3KCA or PTEN is frequently observed in colorectal cancers (31,32). It has been reported that sensitivity to the anti-proliferative effect of AZD6244 was rescued in the resistant cell lines CO115 and DLD-1 when the cells were treated with PI3K inhibitor LY294002 (28). HT-29 cells, but not COLO205 cells, were reported to have a mutation in PI3KCA, suggesting that mutation status in the PI3K pathway may be a possible factor in acquiring resistance to apoptosis. In addition, a recent study demonstrated that GSK1120212 (JTP-74057 DMSO solvate) tends to show cytostatic effects rather than cytotoxic effects in cell lines harboring PI3K or PTEN mutations compared to those of PI3K/PTEN wild-type cells (33). These reports are consistent with the results shown in Fig. 3C, which indicate that the Akt inhibitor API-2 significantly enhanced apoptosis in HT-29 cells treated with JTP-74057. Importantly, GSK1120212 (JTP-74057 DMSO solvate) is being evaluated in a phase I clinical trial in combination with the orally available Akt inhibitor GSK2141795.

Cell proliferation assays have been utilized as a gold standard assay to predict the sensitivity of tumor cells to antitumor agents. However, this may not be sufficient to predict absolute efficacy in vivo. Our observations in this study raise a possibility that an apoptosis assay may also be valuable for prediction of in vivo efficacy with standard cell proliferation assay. Combination therapy is a common approach in cancer chemotherapy. For colorectal cancers, the standard-of-care drugs 5-FU, oxaliplatin and irinotecan are often combined (34). Those agents are known to be highly effective against actively growing cells. In contrast, JTP-74057 induces G_{1} cell cycle arrest in multiple malignant tumor cells. Thus, there was a concern that the efficacy of those standard-of-care drugs might be reduced when JTP-74057 was combined with them. However, JTP-74057 showed an additive effect in combination with 5-FU or oxaliplatin, and a synergistic effect in combination with SN-38. We also examined the combination effect of JTP-74057 with these agents following sequential exposure. JTP-74057 did not show an antagonistic effect in combination when the anticancer drugs were added 24 h after exposure to JTP-74057 or vice versa (data not shown). These results suggest that JTP-74057 may enhance the therapeutic benefit of clinically used anticancer drugs.

In conclusion, we identified a novel MEK1/2 inhibitor JTP-74057 (also called GSK1120212) from a high-throughput screening for compounds that induce expression of p15^{INK4a}. JTP-74057 is selective and orally available. In addition, the antitumor activity of JTP-74057 appears to be superior to those of the second generation MEK inhibitors in preclinical animal models. Furthermore, sensitivity to JTP-74057-induced apoptosis may be an important factor for the estimation of in vivo efficacy, and the sensitivity was enhanced by an Akt inhibitor. GSK1120212 (JTP-74057 DMSO solvate) is now being evaluated in a phase III clinical trial against melanoma patients with B-Raf mutations (NCT01240562), and the results of a phase I study revealed that in 29 B-Raf mutant melanoma patients, 2 complete responses and 6 partial responses were observed (ESMO 2010: http://www.esmo.org/events/milan-2010-congress/news/view.html?tx_ttnews%5Btt\_news%5D=978&tx_ttnews%5BbackPid%5D=585&cHash=86cc01353e). Furthermore, GSK1120212 is also being evaluated in a phase I clinical trials in combination with the Akt inhibitor GSK2141795, the BRAF inhibitor GSK2118436, the mTOR inhibitor everolimus, the PI3K inhibitor GSK2126458 or the PI3K inhibitor BKM120 (ClinicalTrials.gov: http://clinicaltrials.gov/ct2/results?term=GSK2118436). Taken together, JTP-74057 (GSK1120212), originally identified by screening for p15^{INK4a} upregulation, is a promising and novel MEK inhibitor for the treatment of patients suffering from a variety of cancers.

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