Activation of AMP-activated protein kinase by retinoic acid sensitizes hepatocellular carcinoma cells to apoptosis induced by sorafenib

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Key words
AMPK, combination therapy, hepatocellular carcinoma, retinoic acid, sorafenib

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Funding Information
Ministry of Education, Culture, Sports, Science and Technology, Japan.

Received August 31, 2014; Revised January 12, 2015; Accepted February 8, 2015

Cancer Sci 106 (2015) 567–575
doi: 10.1111/cas.12633

Hepatocellular carcinoma is the sixth most common cancer and the third most frequent cause of cancer death worldwide. For patients who have advanced HCC, palliative treatments such as transarterial chemoembolization and sorafenib play a role in improving survival. Multitargeted kinase inhibitor sorafenib is the only drug approved for patients who have either failed transarterial chemoembolization or who present with more advanced HCC. However, sorafenib has been reported to be beneficial in only approximately 30% of patients and acquired resistance often develops within 6 months. Therefore, to improve the outcome of chemotherapy, the strategies that enhance the antitumor effect of sorafenib are urgently required.

Oncogenic mutations can result in the uptake of nutrients, particularly glucose, that meet or exceed the bioenergetics demands of cell growth and proliferation. Cancer cells rely more on glycolysis than oxidative phosphorylation to generate sufficient energy for rapidly proliferating tumor cells, even under normal oxygen concentration. This phenomenon, so-called Warburg effect, has been recognized as one of the hallmarks of cancer, being closely related with either inherent or acquired drug resistance. Therefore, targeting the energy metabolism of cancer cells may be a promising strategy to improve the outcome of chemotherapy. One of the links directly connecting cell metabolism and cancer is AMPK, a central metabolic switch that controls glucose and lipid metabolism. AMP-activated protein kinase becomes activated in response to an increased AMP/ATP ratio, a condition of energetic stress, and promotes the catabolic pathway, inhibiting cell proliferation. Many cancer cells show a loss of appropriate AMPK signaling to overcome this regulation in order to proliferate under abnormal nutrient conditions with an enhanced glycolytic phenotype. Therefore, activation of AMPK is a potential strategy to control tumor cell growth by regulating tumor cell metabolism.

Retinoids, which are vitamin A derivatives, have been reported to prevent cancer in various organs including stomach, breast, lung, prostate, and liver. Retinoic acid, a physiologically active form of retinoid, and its receptor RAR exert their effects by regulating expression of downstream genes in a RARE-dependent manner, namely through retinoid signaling. We previously reported that hepatocyte-specific inhibition of RA signaling caused steatohepatitis, iron accumulation, generation of oxidative stress, and HCC development in the liver of transgenic mice. Furthermore, we showed that retinoids improved insulin resistance, a known...
risk factor for HCC, by restoring leptin signaling in the liver of obesity model mice and HCC cells.\(^{(19)}\) Acyclic retinoid (NIK-333), one of the synthetic retinoids, has been reported to be effective in preventing HCC development in humans and diabetic db/db mice through activating AMPK.\(^{(20,21)}\) These observations helped us to develop the hypothesis that retinoids affect the energy metabolism of cancer cells and show anticancer effects on HCC cells. From this perspective, it is of great interest whether retinoids enhance the cytotoxic effect of anticancer drugs on HCC cells. In this study, we evaluated the effect of retinoids on the cytotoxicity of sorafenib, and found that suppression of glycolysis by retinoic acid sensitizes HCC cells to apoptosis induced by sorafenib through AMPK activation.

Fig. 1. Cell viability assays of HepG2 hepatocellular carcinoma cells treated with retinoids and sorafenib. Cells were treated with sorafenib alone at the indicated concentrations, or in combination with 5 or 10 μM all-trans retinoic acid (ATRA) (a), NIK-333 (b), and Am80 (c) for 48 h. Cell viabilities were determined by WST assay and expressed as percentages of those of control (DMSO treatment). (d) Hepatocellular carcinoma cell lines (PLC/PRF/5, Huh7, HLE, HLF, and Hep3B) were treated with 1 μM sorafenib alone, or in combination with 5 or 10 μM ATRA for 48 h. Cell viabilities were expressed as percentages of those of control (sorafenib alone). *P < 0.05, **P < 0.01 versus control. Experiments were run in triplicate and carried out at least two times on separate occasions.

Fig. 2. All-trans retinoic acid (ATRA) induces AMP-activated protein kinase (AMPK) activation and reduces intracellular ATP content in HepG2 hepatocellular carcinoma cells. (a) HepG2 cells were treated with 0.1 μM sorafenib and 10 μM ATRA alone, or in combination, for 0, 12, 24, or 48 h. Activation of AMPK was detected by immunoblot of phospho-AMPK (Thr-172). (b) HepG2 cells were treated with 0.1 μM sorafenib and 10 μM NIK-333 alone, or in combination. β-Actin served as a control of protein loading. (c) HepG2 cells were treated with 0.1 μM sorafenib and retinoids (ATRA and NIK-333, 10 μM; Am80, 5 μM) for 24 h. Intracellular ATP content was determined by a luciferase-based luminescent assay. *P < 0.05, **P < 0.01 versus DMSO. Experiments were run in triplicate and carried out at least three times on separate occasions.
Fig. 3. Gene expression analysis of enzymes involved in the glycolytic pathway by quantitative RT-PCR. HepG2 hepatocellular carcinoma cells were treated with 0.1% DMSO (D), 0.1 μM sorafenib (S), and 10 μM all-trans retinoic acid (ATRA) alone (A), or in combination (AS), for 24 h. The mRNA expression of glucose transporter 1 (GLUT-1), hexokinase 2 (HK2), glucose-6-phosphate isomerase (GPI), phosphofructokinase, liver (PFKL), aldolase A (ALDOA), triosephosphate isomerase 1 (TPI1), phosphoglycerate kinase 1 (PGK1), phosphoglycerate mutase 1 (PGAM1), enolase 1 (ENO1), pyruvate kinase, muscle (PKM2), and lactose dehydrogenase A (LDHA) was determined by quantitative RT-PCR using gene-specific primers. The levels of mRNA expression were expressed as relative expression to GAPDH mRNA. a, P < 0.05 versus DMSO; b, P < 0.05 versus sorafenib alone. Experiments were run in triplicate and carried out once.
ATRA amplifies drug-induced apoptosis
Materials and Methods

Cell viability assay. Cell viability was determined by the WST assay using Cell Counting Kit-8 (Dojin Chemical Co., Kumamoto, Japan) according to the manufacturer’s instructions.

Western blot analysis. Western blot was carried out using the standard protocol. Primary antibodies used in this study are described in Document S1.

RNA isolation and gene expression analysis. Total RNA was extracted from cells using TRIzol Reagent (Invitrogen, Life Technologies, Tokyo, Japan). Complementary DNA was synthesized by using Superscript II reverse-transcriptase (Invitrogen) and mRNA expression was determined by the LightCycler System using gene-specific primers (Table S1).

Other methods. Additional methods are described in Document S1.

Results

Retinoids enhanced cytotoxic effect of anticancer drugs in HepG2 cells. To examine the effect of retinoids and sorafenib on HCC cell proliferation, cell viability assays were carried out using HepG2 cells. Three retinoids (ATRA, NIK-333, and Am80) and sorafenib significantly reduced cell viability in a dose- and time-dependent manner (Fig. S1). Next, to examine the effect of the combination treatment, HepG2 cells were treated with various concentrations of sorafenib (0–100 μM) alone or in combination with the retinoids. Retinoids were used at the concentrations of 5 and 10 μM, which showed no inhibitory effect on the proliferation of HepG2 cells up to 72 h after treatment (Fig. S1). As shown in Figure 1, ATRA and NIK-333 increased the cytotoxicity of sorafenib, whereas Am80 showed little effect (Fig. 1a–c). The effect of retinoids on the cytotoxicity of anticancer drugs was further investigated in another four drugs, namely adriamycin, mitomycin C, cisplatin, and 5-FU. Although ATRA enhanced the cytotoxic effect of these four drugs, NIK-333 was effective only in one case, when combined with adriamycin (Figs S2, S3). Am80 showed no obvious enhancing effect (Fig. S4). The enhancing effects of combination therapy using ATRA and sorafenib were further confirmed in an additional five HCC cell lines including PLC/PRF/5, HuH6, HLE, HLF, and Hep3B (Fig. 1d). These data suggest that ATRA enhanced the cytotoxic effect of anticancer drugs in HCC cells, being much more effective than NIK-333 and Am80.

All-trans retinoic acid induced AMPK activation and reduced intracellular ATP level of HepG2 cells. To examine the involvement of metabolic modification to the enhancement of cytotoxicity by retinoids, we investigated the activation of AMPK in cells after treatment. As shown in Figure 2, AMPK activation was observed in the cells treated with ATRA alone or in combination with sorafenib at 12, 24, and 48 h after treatment (Fig. 2a). Other than sorafenib, drugs combined with ATRA showed only a minor effect on AMPK activation when the cells were treated with anticancer drugs such as adriamycin, cisplatin, mitomycin C, and 5-FU at concentrations at which their most potent cytotoxicity was observed in the WST assay (data not shown). In addition, AMPK activation was not observed in cells treated with NIK-333 (Fig. 2b). Activation of AMPK has been known to be induced by decreased cellular ATP levels.\(^{7}\) Therefore, we next measured ATP levels in cells treated with retinoids and sorafenib. As shown in Figure 2(c), decreased intracellular ATP levels were observed in cells treated with ATRA, whereas NIK-333 and sorafenib had no effect on ATP levels in either single or combination treatments. These data suggest that ATRA, but not NIK-333, induced AMPK activation by reducing intracellular ATP levels, enhancing the cytotoxic effect of sorafenib.

Gene expression profiles of enzymes involved in glycolysis and TCA cycles. To explore the mechanism underlying the reduction of intracellular ATP by ATRA, mRNA expression of the enzymes involved in glycolysis and TCA cycles was measured by quantitative RT-PCR. Among the glycolytic genes, GLUT-1, TPPI, and LDHA mRNA were significantly downregulated by ATRA treatment compared to DMSO treatment (Fig. 3). GLUT-1, PKM2, and LDHA mRNA were significantly downregulated in the cells treated with the combination of ATRA and sorafenib compared to those of sorafenib alone (Fig. 3). Next, we investigated the mRNA expression of enzymes involved in the TCA cycle. Among the genes, PDK1, PDK2, PDK3, CS, ACO2, IDH1, IDH2, IDH3A, OGDH, and MDH2 were significantly upregulated compared to DMSO treatment (Fig. 4). PDK1, IDH1, and MDH2 mRNA expression was significantly upregulated in cells treated with the combination of ATRA and sorafenib compared to those of sorafenib alone (Fig. 4). In silico analysis revealed that putative RAREs (direct repeat 5) exist in the promoter region 10 kb upstream of these genes (Table S2). These data suggest that ATRA downregulated the expression of glycolytic genes, whereas ATRA upregulated the expression of genes involved in the TCA cycle.

Combined treatment using ATRA and sorafenib induced apoptosis by enhancing intrinsic mitochondrial apoptotic pathway in HCC cells. To investigate the enhancing effect of ATRA on the cytotoxicity of sorafenib in more detail, the number of apoptotic cells was counted. Hoechst staining revealed that apoptosis was increased in cells treated with the combination of ATRA and sorafenib at 24 and 48 h after treatment (Fig. 5a). No induction of apoptosis was observed in cells treated with ATRA or sorafenib alone (Fig. 5b). Treatment with ATRA alone had no inhibitory effect on target kinases of sorafenib including vascular endothelial growth factor receptor-2, c-RAF, MEK, and ERK activation (Fig. S5). Induction of p53 and phospho-p53, a stabilized form of p53, were observed in adriamycin-treated cells (Fig. S6). We next examined the expression of antiapoptotic and proapoptotic proteins by western blot analysis. As shown in Figure 6 (a), upregulation of proapoptotic and proapoptotic proteins by western blot analysis indicated that p38 MAPK and JNK were
Combination treatment of all-trans retinoic acid (ATRA) and sorafenib induced apoptosis by enhancing the intrinsic mitochondrial apoptotic pathway. (a) Western blot analysis of Bcl-xL, Bcl-2, and Bax of cells treated with 0.1 μM sorafenib or 10 μM ATRA alone, or in combination, for 24 or 48 h. β-Actin served as a control of protein loading. (b) Western blot analysis of AMP-activated protein kinase (AMPK) phospho- (p-)AMPK, p38 MAPK, and p-JNK. (c) Identification of subcellular localization of Bax. Cytosolic (Cyto) and mitochondrial (Mito) fractions of the cells were analyzed by Western blot using anti-Bax, anti-α-tubulin (cytosolic marker), and anti-Cox IV (mitochondrial marker) antibodies. (d) Detection of pro-caspase-3 and cleaved caspase-3 in cells at 48 h after treatment. (e) Caspase-3/7 activity of the cells at 48 h after treatment. (f) Effect of AMPK knockdown on the viability of cells treated with sorafenib and ATRA. Upper, two siRNAs were validated for the suppression of AMPK expression. Lower, cells were incubated with control- and AMPK-siRNA for 24 h, and then were subjected to the treatment indicated. WST assay was carried out 72 h after treatment. Experiments were run in triplicate and carried out twice on separate occasions. *P < 0.05, **P < 0.01 (g) Illustrative presentation of the mechanism of additional cytotoxicity induced by ATRA on hepatocellular carcinoma cells treated with sorafenib.

Discussion

In this study, we investigated the enhancing effect of retinoids on the cytotoxicity of sorafenib in HCC cells. Cell viability assays showed that the potency of the enhancing effect proved to be different, depending on the type of retinoids. As retinoids exert their effect through their binding to RARs or RXRs, their biological effects largely depend on the selectivity of receptors. All-trans retinoic acid is a natural ligand of all RAR isoforms (α, β, and γ) and is metabolized to 9-cis RA, which activates both RAR and RXR in the cells. NIK-333 activates RXRα with its agonistic activity and, additionally, is shown to restore the function of RXRα by inhibiting Ras–Erk signaling-mediated phosphorylation, which inactivates RXRα. Am80, a RARα/β-selective retinoid that does not bind and activate RARγ or RXR, showed no enhancing effect in this study. In pancreatic cancer cells, only pan-RAR and RARγ-selective agonists were shown to reduce cell viability. In combination with anticancer drugs, the enhanced cytotoxic effect on pancreatic cancer cells was observed in ATRA, 9-cis RA, and NIK-333. These reports and our data suggest that activation of the retinoid signal by RARγ and RXR plays an important role in this effect.

In the combination treatment, 5 and 10 μM ATRA showed similar levels of additional cytotoxicity. Similarly, the cytotoxicity of ATRA alone did not differ between 5 and 10 μM (Fig. S1). The level of AMPK activation was comparable between 5 and 10 μM ATRA (data not shown). Therefore, it is reasonable that the additional cytotoxic effect of ATRA did not differ among these concentrations.

To clarify the mechanism of the enhancing effect by ATRA, we focused on the energy metabolism of HCC cells. It is known that AMPK can function as an intracellular energy sensor, being activated when the cells meet the condition of energetic stress, such as ATP depletion. Among these concentrations. Activated AMPK can activate both RAR and RXR in the cells. NIK-333 activates RXRα with its agonistic activity and, additionally, restores the function of RXRα by inhibiting Ras–Erk signaling-mediated phosphorylation, which inactivates RXRα. Am80, a RARα/β-selective retinoid that does not bind and activate RARγ or RXR, showed no enhancing effect in this study. In pancreatic cancer cells, only pan-RAR and RARγ-selective agonists were shown to reduce cell viability. In combination with anticancer drugs, the enhanced cytotoxic effect on pancreatic cancer cells was observed in ATRA, 9-cis RA, and NIK-333. These reports and our data suggest that activation of the retinoid signal by RARγ and RXR plays an important role in this effect.
suggest that ATRA reduces ATP production by inhibiting the energy-producing pathway in HCC cells. In the gene expression analyses, we found that ATRA downregulates the expression of GLUT-1 and LDHA, two important genes for glycolysis. The rate of glucose metabolism is regulated by glucose transport by Glut-1 and NAD+ recycling catalyzed by LDHA. Therefore, the crucial role of these genes for ATP production and cancer cell growth were reported in various types of cancer, including HCC. Together, the downregulation of GLUT-1 and LDHA by ATRA may be responsible for the reduction of ATP and enhancement of the antitumor effect of sorafenib in HCC cells.

Downregulation of LDHA promotes the entry of pyruvate into mitochondria to form acetyl-coA, a substrate for the TCA cycle and oxidative phosphorylation. As cancer cells have been reported to have functional mitochondria, acetyl-coA is catabolized to yield ATP efficiently by oxidative phosphorylation in the presence of oxygen. Conversion of pyruvate to acetyl-coA is catalyzed by PDH, whose activity is negatively regulated through phosphorylation by PDKs. As ATRA upregulated PDK1, PDK2, and PDK3, which inactivates PDHA, carbon influx into the TCA cycle may be suppressed. This action leads to reduction in compensational ATP production from mitochondria when glycolytic ATP production is lowered.

The mechanism of downregulation of GLUT-1 and LDHA expression by ATRA remains to be further investigated. We found that putative RARE motifs are present in the upstream region of GLUT-1 and LDHA genes (data not shown). Therefore, direct regulation by ATRA in a RARE-dependent manner may exist. Alternatively, indirect regulation mediated by hypoxia-inducible factor-1α, a major inducer of glycolytic genes, may be involved in this phenomenon. All-trans retinoic acid upregulated IDHs, which catalyze the conversion of isocitrate to α-ketoglutarate in the TCA cycle. Therefore, activation of the α-ketoglutarate-dependent prolyl hydroxylase (PHD) may promote hypoxia-inducible factor-1 degradation. These mechanisms need to be further investigated to understand the glycolytic inhibition by ATRA.

Although AMPK is mainly considered to be a pro-survival kinase that promotes the catabolic pathway, its involvement in the induction of cell death has also been established, mostly in conditions of its sustained activation. It has been reported that p38 MAPK is a downstream substrate of the AMPK–MAPK kinase (MKK) axis, involving Bax translocation and induction of mitochondrial apoptosis. Moreover, activated p38 and JNK are reported to phosphorylate Bax and promote its conformational change, which facilitates translocation from the cytosol to mitochondria. Uptregulation and activation of Bax were observed in the cells undergoing apoptosis induced by treatment with sorafenib or retinoids. This mechanism is consistent with the hypothesis that enduring bio-energetic crisis is a crucial factor that couples energy stress with apoptosis induction.

All-trans retinoic acid had an additional cytotoxic effect on five anticancer drugs, namely sorafenib, adriamycin, cisplatin, mitomycin C, and 5-FU, but the level of cytotoxicity was different among these agents. In sorafenib treatment, Bax upregulation and translocation to mitochondria were the key events for additional cytotoxicity of ATRA. As ATRA sensitized cells to the mitochondria-mediated intrinsic apoptosis pathway, we examined the expression of p53, an important regulator of mitochondria-dependent cell death, in cells treated with these agents. Induction of p53 and phospho-p53, a stabilized form of p53, were observed in adriamycin-treated cells (Fig. S6). In mitochondria, p53 binds anti-apoptotic Bel-2 and Bel-xL, releasing the pro-apoptotic Bax–Bak complex and triggering apoptosis. Therefore, the cytotoxicity of adriamycin could be potentiated by ATRA treatment. Activation of caspase-8 and caspase-3 was reported to play a role in the apoptosis of mitomycin C-treated hepatoma cells. As caspase activation promotes Bax conformational change and its mitochondrial translocation, the cytotoxicity of mitomycin C may also be enhanced by ATRA. In cisplatin and 5-FU treatment, high concentrations such as 10 μM, where substantial apoptotic signals were exerted, were effective in showing the additional cytotoxicity of ATRA.

In conclusion, we showed the enhanced cytotoxic effects of sorafenib in cells treated with retinoids. Downregulation of glycolytic genes and reduced ATP production by ATRA induced AMPK activation and promoted the mitochondrial apoptotic pathway. These mechanisms provide an opportunity to improve the efficacy of chemotherapy by regulating the metabolic pathway crucial for the survival of cancer cells.

**Acknowledgment**

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

**Disclosure Statement**

The authors have no conflict of interest.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| AMPK         | AMP-activated protein kinase |
| ATRA         | all-trans retinoic acid |
| 5-FU         | 5-fluorouracil |
| HCC          | hepatocellular carcinoma |
| IDH          | isocitrate dehydrogenase |
| LDHA         | lactate dehydrogenase A |
| PDH          | pyruvate dehydrogenase |
| PDK          | pyruvate dehydrogenase kinase |
| RA           | retinoic acid |
| RAR          | retinoic acid receptor |
| RARE         | retinoic acid response element |
| RXR          | retinoid X receptor |
| TCA          | tricarboxylic acid |

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Putative retinoic acid response elements (RAREs) found in the promoter region (Table S2).

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Fig. S2. Supporting Information

Cell proliferation assay of HepG2 cells treated with retinoids and sorafenib.

Fig. S1.

Fig. S6. Cell viability assays of HepG2 cells treated with a combination of anticancer drugs and Am80.

Fig. S5.

Fig. S4. Western blot analyses of phospho-vascular endothelial growth factor receptor 2 (VEGFR2), phospho-c-Raf, phospho-MEK1/2, and phospho-ERK in HepG2 cells treated with a combination of sorafenib and all-trans retinoic acid (ATRA).

Fig. S6.

Table S1. Primers for quantitative RT-PCR analysis.

Table S2. Putative retinoic acid response elements (RAREs) found in the promoter region (−10 kb) of glycolytic and tricarboxylic acid cycle genes.