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Original Article

Combination of autocrine motility factor and resveratrol enhances inhibition of HL-60 human leukemic cells

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ABSTRACT

Autocrine motility factor (AMF) is well known for its ability to promote the motility, proliferation, and metastasis of cancer cells. However, extracellular AMF has been found to selectively kill cancer cells in an AMF type- and dose-dependent manner. In this study, the effects of eight different AMFs on HL-60 human leukemic cells were investigated. The AMF derived from HepG2 liver cancer cells (HG:AMF) was found to be more aggressive than the other AMFs and was further investigated for its inhibition of HL-60 cells in a dose- and time-dependent manner. Prolonged treatment with higher concentrations of HG:AMF was found to be severely detrimental to HL-60 cells. The inhibition of HL-60 cells by HG:AMF was visible under the microscope, as formazan deposits appeared in the sphere of aggregated HL-60 cells. HG:AMF was also found to significantly enhance ERK1/2 phosphorylation in a dose-dependent manner, while upregulating pro-apoptotic Bax and cleaved caspase 3 expression. No change was detected in the level of phosphorylated AKT and p70S6K, as well as anti-apoptotic Bcl-2. In a combination study, resveratrol (RV) was found to exert a cooperative and synergistic effect against the growth of HL-60 cells, while downregulating AKT activation. Cooperation with tamoxifen, a selective estrogen receptor modulator, was also suggested. Thus, HG:AMF alone or in combination with RV is recommended as a promising option for human leukemia therapy with the least risk of generating resistance.

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KEYWORDS: acute promyelocytic leukemia, apoptosis, autocrine motility factor, HL-60, resveratrol.

INTRODUCTION

Acute promyelocytic leukemia (AML) is a malignant neoplasm of the hematopoietic stem cells. Despite significant progress in AML chemotherapy, the long-term survival of AML patients remains poor mainly due to the development of resistance to chemotherapeutic agents [1]. As a promising alternative, biotherapeutic proteins and monoclonal antibodies have received increased attention, but their practical applications in AML therapy are yet to be fully explored [2].

Many moonlighting proteins, which are involved in cellular proliferation and metabolism, exhibit different functions when targeted to different subcellular localizations. Moonlighting activity is typically observed when there are changes in temperature, redox potential, oligomerization, and binding partner proteins [3]. Some moonlighting proteins have opposing effects on cell survival and death, including members of the Bcl-2 family [4]. Glucose 6-phosphate isomerase (GPI) is primarily utilized for converting glucose-6-phosphate and fructose-6-phosphate in the glycolysis and gluconeogenesis pathway [5]. GPI has a moonlighting role [6]. When secreted, GPI acts as both autocrine motility factor (AMF) and neuroleukin (NLK). NLK induces immunoglobulin secretion and promotes the survival of different types of neurons [7], whereas cancer cell-secreted AMF cytokine exhibits motogenic

and mitogenic activity through an autocrine route [8]. AMF is known to activate the PI3K/AKT and/or MAPK/ERK signaling pathways, hindering apoptosis and mitigating ER stress response in cancer cells [9-11]. Aberrant secretion of AMF is frequent in various human cancers. Previously, we reported that AMF can act against the proliferation of lung [12], breast [13], and liver [14] cancer cells, despite its beneficial effect on cancer cell survival and cancer progress. Furthermore, we proposed that extracellular AMF might act as a soluble killing signal to trigger the process of cell competition and eradication. AMF is like a double-edged sword that might exert a vital force in determining cancer cell survival or death during competition [15, 16]. Additionally, it should be emphasized that extracellular AMF, identical to cytosolic GPI, would be freely allowed for reentry without triggering vigilance in cancer cells. Therefore, understanding the effect of AMF on the proliferation of HL-60 cells might provide a novel approach to develop AML biotherapy without the risk of drug resistance.

Natural products have emerged as promising sources for seeking safe and effective anti-cancer drugs. Numerous phytochemicals exhibit anti-cancer properties by targeting multiple signaling pathways. Resveratrol (RV) is widely present in various plants and synthesized in response to pathogenic infection, mechanical injury, or abiotic stress such as ultraviolet radiation [17]. RV has several beneficial effects on human health and has been found to be effective in many cancer cells, such as lung, melanoma, neuroblastoma, prostate, breast cancer, and HL-60 cells [18]. It has been strongly suggested that RV may play a synergistic role with other anti-cancer agents targeting non-overlapping cellular pathways [19].

Based on our previous findings [12-14], we expected different AMFs to differentially affect the proliferation of HL-60 AML cells. Indeed, HG:AMF was highly suppressive on HL-60 cell growth, upregulating ERK phosphorylation possibly above a threshold level. Furthermore, HG:AMF showed its efficacy in sensitizing HL-60 cells to cytotoxic RV. Considering that cancer cells are always in need of AMF for motility, proliferation, and anti-apoptosis, HL-60 cells may never be able to sense HG:AMF's hostility until death in the absence of resistance.

MATERIALS AND METHODS

Cell culture and growth assay

HL-60 cells were obtained from the Korea Cell Line Bank (Seoul, Korea) and were cultured in RPMI medium containing Gibco GlutaMAX^{TM}, 10% fetal bovine serum (FBS), and penicillin/streptomycin antibiotics at 37 °C in a 5% CO_2 humidified incubator. For the initial plating after thawing, HL-60 cells were supplemented with 20% FBS. In a short-term (ST) treatment, 1.2×10^4 HL-60 cells in 200 μL of medium were plated in each well of 96-well round-bottom culture plates and subjected to treatment with AMF for 48-72 h. In a long-term (LT) treatment, 0.5×10^4 cells were similarly treated for 7 d. In a combination assay, AMF was treated at least 2 h prior to the addition of RV, methyl jasmonate, tamoxifen, or sorafenib at various concentrations. To measure cell growth, cells were treated with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) for 3 h. Culture plates were centrifuged at 3000 rpm for 10 min, the medium was carefully removed by aspiration, and then a DMSO/methanol (1:1) solvent was added. Samples were transferred to a flat-bottom microplate and then monitored at 570 nm.

Recombinant AMF proteins

Recombinant AMFs were obtained from various cancer cell lines, including AsPC-1 pancreatic cancer cells (AP:AMF, Genbank: MW664917), DU145 prostate cancer cells (DU:AMF, Genbank: MW664916), HeLa cervical cancer cells (HLa:AMF, Genbank: KY379509), HepG2 liver cancer cells (HG:AMF, Genbank: MW664918), HT29 prostate cancer cells (HT:AMF, Genbank: MW843569), MCF-7 breast cancer cells (M7:AMF, Genbank: MW664919), and SKOV3 ovarian cancer cells (SK:AMF, Genbank: MW664910). The AMF from A549 cells is identical to human GPI (A:AMF, Genbank: BC004982). To prepare recombinant AMF proteins, a modified version of the previously described procedure [12] was used. *Escherichia coli* BL21 cells containing AMF cDNA in the pCold I DNA plasmid vector were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 24 h at 15 °C as per the manual of the pCold I DNA cold-shock expression system. The bacterial cells were then lysed on ice for 30 min using extraction buffer (50 mM sodium phosphate

pH 8.0, 300 mM NaCl, 10 mM imidazole, and 0.5 mM PMSF) containing 1 mg/mL lysozyme. The samples were sonicated (six 15 sec bursts at 250 W with a 15 sec cooling between each burst) and then centrifuged at 13,000 rpm for 20 min at 4 °C. The cleared lysates were filtered through a 0.22 μm syringe filter and then applied to His60 Ni resin affinity chromatography (Promega, Madison, WI). The samples were concentrated using a spin column (MWCW:10K), and AMF proteins were quantified using the Bio-Rad protein assay reagent.

Apoptosis analysis

Cell apoptosis was analyzed using the Muse® Annexin V & Dead Cell Kit from Millipore Co. (Billerica, MA, USA) following the manufacturer's instructions.

Western blot analysis

Cells grown in 100 mm culture dishes were washed with PBS and then lysed on ice for 30 min in RIPA buffer containing 1 mM β-glycerophosphate, 5 mM potassium fluoride, 0.1 mM sodium orthovanadate, and protease inhibitor cocktails. Cell lysate (20 μg total) was separated on a sodium dodecyl sulfate (SDS) polyacrylamide gel, transferred to a polyvinylidene difluoride (PVDF) membrane, and then subjected to Western blot analysis using an enhanced chemiluminescence (ECL) system. Experimental results were obtained using a Microchemi™ imaging system (Bio-Imaging System, Neve Yamin, Israel). Antibodies against β-actin, pAKT, AKT, pERK1/2, ERK, p70S6K, p-p70S6K, Bcl-2, Bax, HIF-1α, and CHOP were purchased from Cell Signaling Technology (Danvers, MA).

Statistical analysis

The results are presented as the mean ± standard deviation (S.D.). Statistical significance was determined using Student's t-test for comparison between two groups. For multiple comparisons, a one-way analysis of variance (ANOVA) F-test was used. A significance level of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

In an ST growth assay for 48 h, HL-60 cells were treated with eight different AMFs (Fig. 1a). At a concentration of 0.5 μg/mL, A:AMF had little effect, while other AMFs reduced growth to 86-94% of untreated control cells (Fig. 1b). We hypothesized that cancer cell viability might be altered depending on the outcome of the interaction between different AMFs. Therefore, we mixed AMFs using non-harmful A:AMF, less harmful AP:AMF, and aggressive HLa:AMF and HG:AMF. The results showed that A:AMF appeared to counteract the activity of other AMFs and relieve cell suppression. Additionally, HG:AMF and HLa:AMF in combination appeared

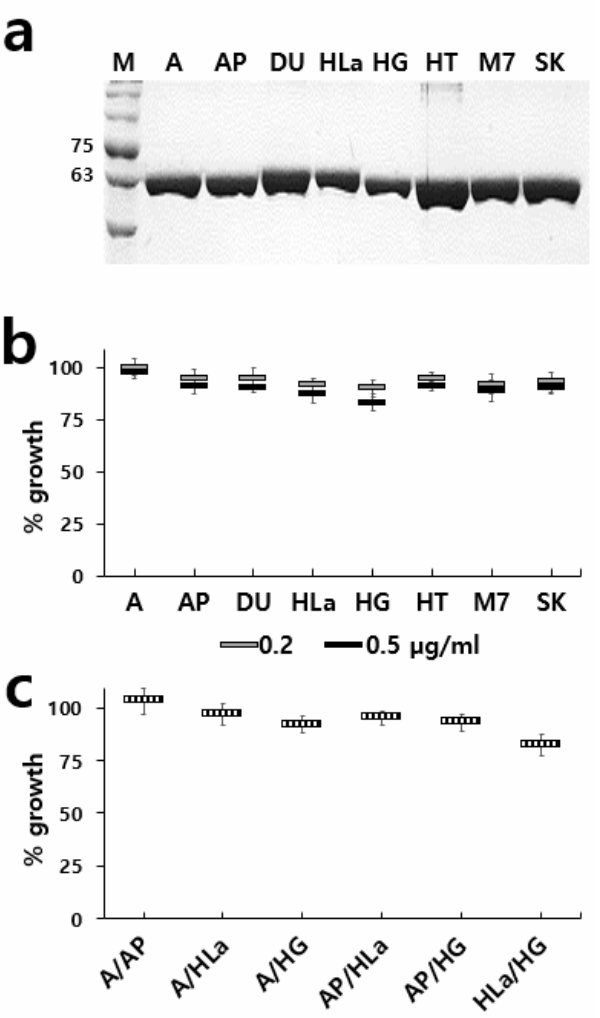

Fig. 1. Differential inhibition of HL-60 human leukemic cells by various AMFs. (a) Coomassie blue staining of 0.5 - 1 μg of eight different recombinant AMFs separated by SDS-PAGE. (b) The growth of HL-60 cells after treatment for 48 h with 0.2 and 0.5 μg/mL AMF. (c) The growth of HL-60 cells after combined treatment of two different AMFs for 48 h with 0.5 μg/mL. Data are presented as mean ± SE.

to exert a suppressive force stronger than each alone (Fig. 1c), suggesting that competitive and cooperative AMF interactions trigger the process of cellular fitness sensing in cancer cell competition [15, 16].

We further assessed HG:AMF due to its distinctive aggressiveness. As expected, it inhibited HL-60 cells in a dose- and time-dependent manner. In an ST treatment for 72 h, 0.2, 0.5, 1, and 2 μg/mL of HG:AMF reduced the growth of HL-60 cells to 95, 87, 82, and 78% of that of untreated control cells, respectively (Fig. 2a). At concentrations of 0.2 and 0.5 μg/mL, the results of LT treatment were not significantly different from those of ST treatment. However, LT treatment at higher concentrations was severely harmful, reducing growth to 60 and 41% at 1 and 2 μg/mL, respectively. Considering that cancer cells may require 20-500 nM AMF for their migration and

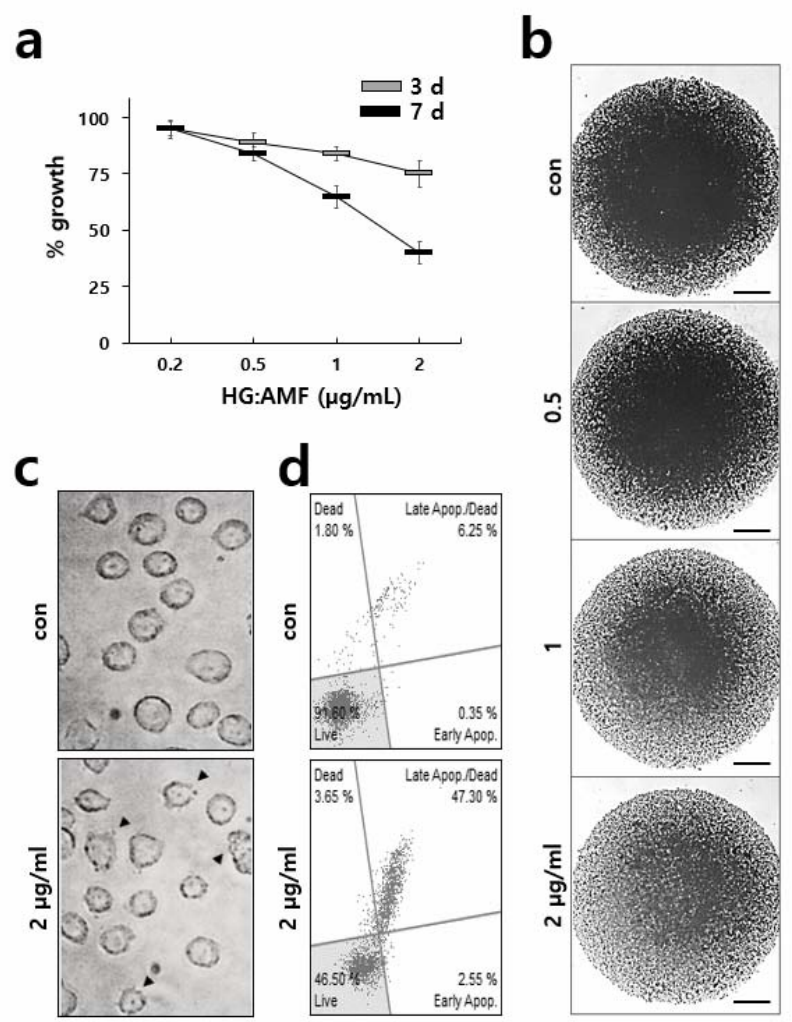

Fig. 2. HG:AMF induces apoptosis and impairs growth in HL-60 cells. (a) Comparative measurement of viable HL-60 cells was performed after treatment with HG:AMF at various concentrations for 3 and 7 d. (b) HL-60 cells were treated with HG:AMF for 48 h and then incubated with MTT solution for 3 h. Aggregated HL-60 cells in a spherical shape were monitored under the microscope. The original magnification of the images was 50× and the size bar indicates 250 μm. (c) Morphological changes in HL-60 cells (as indicated with arrows) were microscopically observed after treatment for 24 h with 2 μg/mL HG:AMF. The original magnification of the images was 50×. (d) HL-60 cells in apoptosis were measured after treatment with 2 μg/mL HG:AMF for 48 h. Data are presented as mean ± SE.

proliferation [8, 20], HL-60 cells might willingly permit HG:AMF's entry without expressing reluctance.

In a round-bottom culture plate, HL-60 cells were able to form 3D-like spheres in suspension, which allowed us to monitor changes in cell growth and viability under a microscope. The effects of HG:AMF on HL-60 cell viability were visualized through the reduction in formazan deposits in a dose-dependent manner, without any significant difference in the size of the spheres between treated and untreated control cells (Fig. 2b). This was further supported by the irregular morphology (Fig. 2c) and apoptotic progression (Fig. 2d) observed in HG:AMF-treated cells, indicating that HG:AMF effectively induced apoptotic death of HL-60 cells by reducing cell viability while still allowing for moderate cell division.

In recent years, combining phytochemicals with chemotherapeutic agents has emerged as a promising approach to cancer therapy due to its low toxicity and resistance [21]. However, there are only a few reports on the use of phytochemicals in combination with biotherapeutic agents. Resveratrol (RV) is known for its potent anti-proliferative effects on cancer cells, including HL-60 cells. It can induce intrinsic and extrinsic apoptosis and cell cycle arrest, and inactivate the PI3K/AKT/mTOR signaling pathway [17, 18]. In this study, RV alone significantly impaired the growth of HL-60 cells (Fig. 3a). After an ST treatment for 72 h, RV at concentrations of 10, 20, and 30 μM led to a growth decrease of 6, 34, and 79%, respectively. Similarly, an LT treatment resulted in a decrease of 47, 67, and 97%, respectively. In combination studies, we found that HG:AMF treatment for at least 2 h prior to RV treatment produced greatly improved results with high consistency, whereas simultaneous treatment did not have a consistent combination effect. In an ST treatment for 72 h, 0.5 μg/mL HG:AMF and 10 μM RV induced a 23% decrease in cell growth, whereas each alone caused a 11% and 6% decrease, respectively. Similarly, 1 and 2 μg/mL HG:AMF with 10 μM RV induced a 40 and 50% decrease, respectively, whereas each alone resulted in an 18 and 20% decrease, respectively (Fig. 3b). In an LT treatment, a co-treatment of 0.2 μg/mL HG:AMF and 10 μM RV induced a 60% decrease in cell growth when each alone causing a 5% and 47% decrease, respectively. Similarly, with 10 μM RV, a 69, 80, and 95% cell decrease at 0.5, 1, and 2 μg/mL HG:AMF in combination, respectively was induced while each alone caused a 16, 35, and 55% decrease, respectively (Fig. 3c). A synergy between HG:AMF and RV seemed to occur at relatively lower concentrations. The synergistic effect was also confirmed by monitoring the size and formazan deposit lightness in the sphere, as presented in Fig. 3d. It was of interest to investigate whether HG:AMF could sensitize other anti-cancer agents, such as methyl jasmonate (MJ), a cytotoxic plant hormone [13, 22], tamoxifen (TAM), a selective estrogen receptor modulator [23], and sorafenib (SOR), a protein kinase inhibitor [24] that is used in AML chemotherapy. The results showed that HL-60 cell inhibition induced by MJ, SOR, and TAM was relieved, unaffected, and enhanced, respectively, by the combination with HG:AMF (Fig. 3e). These findings suggest that combining AMF properly with phytochemo- or chemotherapeutic agents could be an attractive strategy for fighting cancers, including AML.

Receptor tyrosine kinases play a critical role in governing the proliferation of cancer cells. Among them, FMS-like tyrosine receptor kinase 3 (FLT3) inhibitors have emerged as one of the primary chemotherapeutics for AML. Altered expression of FLT3 can disturb various signaling pathways including Ras/Raf/MEK/ERK, PI3K/AKT, and STAT5, which can lead to growth factor independence, cellular proliferation, and chemoresistance [24-26]. AMF can activate these signaling pathways by binding to its receptors, such as AMFR, HER2, and G protein-coupled estrogen receptor (GPER) [9, 10]. Furthermore, AMF can induce cancer cell apoptosis by regulating AKT and/or ERK signaling activity in a dose- and cancer cell type-dependent way [12-14]. We hypothesized that such signaling activities, possibly governed by FLT3 in HL-60 cells [25], could be altered upon treatment with HG:AMF. However, treatment with HG:AMF at varied concentrations for 24 h did not change the level of phosphorylated AKT and HIF-1α, a downstream target of AKT signaling. Despite the highly activated AKT, the level of cleaved caspase 3 was increased by HG:AMF in a dose-dependent manner, indicating the death of HL-60 cells through pathways other

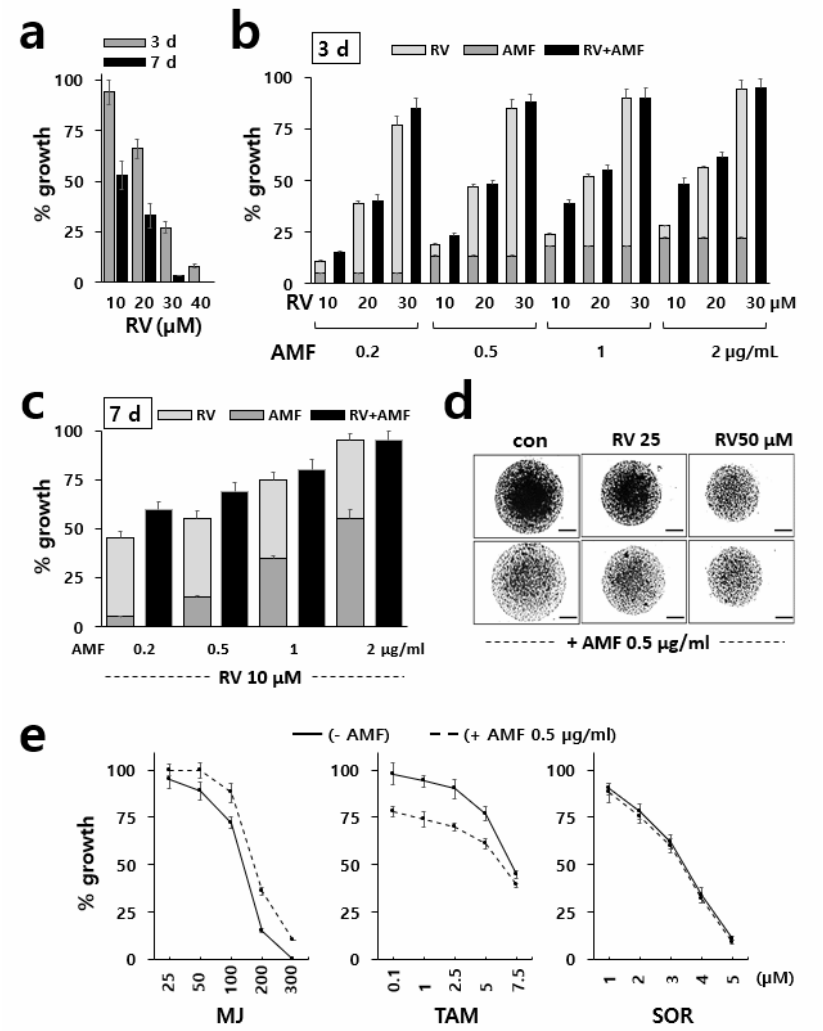

Fig. 3. Synergistic inhibitory effects of HG:AMF in combination with RV on HL-60 cell growth. (a) HL-60 cells were treated with RV for 3 and 7 d at various concentrations. HL-60 cell growth was compared after RV and HG:AMF were co-treated for 3 d (b) and 7 d (c). (d) After treatment with HG:AMF and RV for 48 h, HL-60 cells were incubated with MTT solution for 3 h and monitored under the microscope. The size bar indicates 250 μm. (e) The growth of HL-60 cells was compared following treatment for 48 h with MJ, TAM, and SOR each alone or along with HG:AMF. Data are presented as mean ± SE.

than AKT signaling (Fig. 4a). In contrast, ERK1/2 phosphorylation was remarkably enhanced by HG:AMF in a dose-dependent manner (Fig. 3b). The 70 kDa ribosomal protein S6 kinase (p70S6K) is a downstream target of AKT, but its regulation by ERK signaling has been controversial from cell type to cell type [27]. In HL-60 cells, HG:AMF-induced ERK activation did not alter the level of p70S6K activation. Anti-apoptotic Bcl-2 is one of the most promising targets for AML therapy due to its significant contribution to chemoresistance [28]. However, HG:AMF failed to impede the level of Bcl-2. Nevertheless, the level of Bax was significantly increased along with the increase in ERK1/2 phosphorylation, indicating that the Bax and Bcl-2 ratio is significantly associated with HG:AMF-induced HL-60 cell inhibition. RV is known for its ability to downregulate AKT activation [17, 18]. Treatment of HL-60 cells with 30 μM RV for 24 h significantly reduced AKT phosphorylation (Fig. 4c). Interestingly, AKT activation appeared to be further inhibited by RV in the presence of

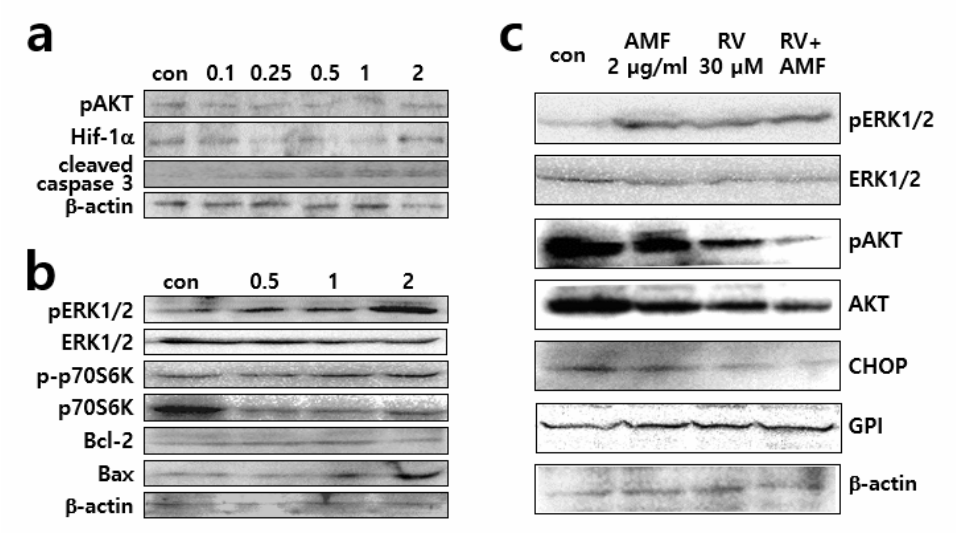

Fig. 4. Inhibition of HL-60 cells by HG:AMF-induced ERK activation alone and in combination with RV-induced AKT inactivation. (a) HG:AMF did not affect AKT phosphorylation but increased cleaved caspase 3. (b) HG:AMF upregulated ERK1/2 phosphorylation and Bax but did not affect the expression level of Bcl-2. (c) RV downregulated AKT phosphorylation but did not affect ERK1/2 phosphorylation. HG:AMF and RV synergistically downregulated AKT activation.

HG:AMF, despite no direct effect of HG:AMF on AKT signaling, as shown in Fig. 4a. Additionally, RV slightly upregulated ERK activation, while HG:AMF-induced ERK activation was still maintained even in the presence of RV. These findings suggest that the combined treatment of RV and HG:AMF induces HL-60 AML cell death through the concerted activity of non-overlapping cellular signaling pathways. RV-induced decrease of CHOP, an ER stress marker protein, may contribute to further cellular damage, while the glycolytic activity of cytosolic GPI is not affected by either RV or HG:AMF.

Chemotherapy has been the traditional treatment for AML patients, but targeted therapies have gained prominence in recent years. These therapies aim to selectively target mutated molecules, such as FLT3, isocitrate dehydrogenase, and Bcl-2, as well as inhibitors of the hedgehog pathway. However, primary and acquired resistance remains a significant challenge. Drug resistance may be inevitable unless anti-cancer drugs can be recognized by cancer cells as self-molecules. This is why human-derived cytotoxic proteins, such as tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), have emerged as a promising alternative with potentially lower incidence of resistance. TRAIL induces cancer cell death while also engaging the human immune surveillance system to recognize mutated cancer cells as non-self. However, cancer cells can develop resistance to TRAIL after prolonged exposure, possibly due to the ability of cancer cells to recognize a non-self aspect hidden in the immune surveillance system that employs TRAIL. Moreover, many cancer cells exhibit intrinsic resistance to TRAIL, suggesting that mutated cancer cells have genetically evolved adaptations to this cytotoxic protein as it travels through the bloodstream.

The discovery that HG:AMF induces ERK activation and cell death in HL-60 cells is noteworthy, as little is known about the mechanism of AMF-induced cell death. AMF is secreted from cancer cells and can re-enter through its receptor, allowing it to contribute to various aspects of cancer progression, such as migration, proliferation, anti-apoptosis, resistance, and angiogenesis, *via* autocrine and/or paracrine routes. Despite extensive research on the underlying mechanism of AMF, the killing action mechanism has received limited attention. In general, ERK signaling must be activated to a certain level to drive cancer cell survival and proliferation. ERK signaling activities below or above a threshold level can induce cancer cell

death through autophagy, senescence, apoptosis, and pinocytosis. Depending on the type of cancer cell, ERK activation may result in the upregulation of Bax and cleaved caspase-3 expression, as demonstrated in this study. This suggests that HG:AMF may act as a "killing spy" equipped with a double-edged sword by delicately regulating ERK activation *in vivo* and *in vitro*. The differential inhibition of HL-60 cells by different types of AMFs may reflect their distinct modes of action in competitive environments where cancer cells struggle to survive. Many anti-cancer drugs have been shown to kill cancer cells while inducing ERK signaling activation, indicating that the ERK activation cascade ultimately disrupts the activity of transcription factors, such as FOS, Elk-1, Jun, ETS, and Myc, which promote cancer cell survival and proliferation.

RV is a natural constituent of wine, grapes, and other edible fruits that is well-tolerated and considered a safe part of the human diet. However, its low bioavailability and lipophilic properties can be improved in the near future [17, 18]. RV has been found to inhibit the proliferation of HL-60 cells by inducing apoptosis, S-phase cell cycle arrest, and myelomonocytic differentiation. Because of RV's ability to regulate LKB1-AMPK and PI3K/AKT signaling pathways and other oncoactivities, HG:AMF was deemed an ideal partner to cooperatively eliminate HL-60 cells while minimizing the dosage and resistance of each other. The results obtained using both agents, even at low concentrations, were as expected. Based on the results of combination studies, we strongly believe that AMF could be used as an effective sensitizer to treat AML with selected anti-cancer phytochemo- and chemotherapeutic agents. Despite their high metabolic plasticity and proficient resistance, HL-60 cells may not be able to recognize the threat posed by AMF, which could be perceived as a self-molecule. Concomitant use of RV can accelerate the death of HL-60 cells.

CONCLUSION

It is suggested that AMF, either alone or in combination with RV, may offer a promising and cost-effective alternative for AML therapy, while also reducing the risk of drug resistance development.

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CONFLICT OF INTEREST STATEMENT

The authors have no potential conflict of interest to report.

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