may enhance development of compounds that may serve as a new class of anticancer drugs which regulate the tumor metabolome.

An important question raised by our results is how the decrease in cellular ATP caused by the inhibition of glycolysis and mitochondrial respiration by GPA and PA co-treatment results in suppression of EGF-stimulated filopodia protrusions in A431 cells. The molecule Profilin may hold the key to this answer. Profilin is critical role for filopodia protrusion because it promotes polymerization of filamentous actin (F-actin) at the extending ends of filopodia (Witke, 2004).

Profilin binds to ADP-bound actin monomers, promotes exchange of ADP for ATP, and releases ATP-actin at the growing ends of F-actin, leading to polymerization of actin in a straight-lined form (Le Clainche and Carlier, 2008; Witke, 2004). The profilin-mediated elongation of F-actin bundles pushes the cell membrane outward, resulting in the protrusion of spike-shaped filopodia. In this regard, ATP provides the energy for Profilin-mediated filopodia protrusion. Moreover, Molitoris *et al.* observed that ATP depletion resulted in punctate dispersion of F-actin from its straight-lined form (Molitoris, et al., 1991),

suggesting that stabilization of straight-lined F-actin depends on the level of ATP in the cell. Therefore, one possible explanation for the inhibition of filopodia protrusion by GPA and PA co-treatment might be the lowering of intracellular ATP concentration caused by the blockage of glycolysis by GPA and mitochondrial respiration by PA.

Our study was based on the screening of crude natural products and bioassay-guided isolation of the components that inhibit filopodia protrusion. Recently, natural product screening has declined in popularity, probably because the isolation and structural determination steps are costly and time-consuming. However, we believe our use of this technique to isolate compounds that act synergistically to inhibit an important cellular process demonstrate that crude natural product screening is still a valuable technique. Since cellular responses are driven by many complex systems that are often robust due to the presence of rescue and feedback pathways, the best strategy for finding bioactive inhibitors of a particular cellular system may be global screening of crude extracts of natural products. In conclusion, though the approach in this study

may be deemed "old-fashioned" and somewhat laborious, we believe the results provided here have opened the broad avenue of natural products screening for the continued progress in chemical genetics research.

Significance

This study began with the natural product screening to obtain the unique bioactive compounds. To obtain the unique bioactive compounds, filopodia seem a good target for the inhibitor screening from microbial origin because of few inhibitors in reports. Moreover, filopodia in tumor cells contribute to the metastasis, therefore, such inhibitor holds the therapeutic impacts for the tumor treatment. By screening the microbial broth, we found the cultured broth of one *Lechevalieria sp.* strain that inhibited the tumor filopodia protrusion. However, this inhibition disappeared following silica-gel chromatography. Interestingly, the inhibitory activity was almost completely recovered by re-mixing all of the silica-gel chromatography fractions, suggesting that the inhibition required the synergistic effect of two or more compounds contained within the microbial broth that eluted in different fractions. We tried to isolate the components responsible for inhibition of filopodia protrusion and found glucopiericidin A (GPA) and piericidin A (PA). PA is a known inhibitor of mitochondrial respiration, but the mode of action of GPA has not yet been reported. Our experiments with

CE-TOFMS metabolomic analysis showed that GPA suppressed glycolysis and identified glucose transporters as the functional target molecule of GPA. Importantly, GPA is glucopyranoside derivative of mitochondrial respiratory inhibitor PA. This glycosidation of PA into GPA lost the inhibitory activity to mitochondrial respiration but gained the inhibitory activity to glucose uptake, which would be informative to chemists to control the inhibitor target between glucose transporter and mitochondrial respiration by a simple glycosidation. Finally, we found that GPA-mediated inhibition of glycolysis dramatically decreases intracellular ATP levels only when mitochondrial respiration is inhibited, and concluded that this ATP decrease caused the synergistic filopodia inhibition by GPA and PA. In the end, this is the first report on the novel use of CE-TOFMS metabolomic analysis to isolate the target protein of the natural product inhibitor.

Experimental Procedures

Filopodia protrusion assay and ATP determination

Cells were seeded sparsely at 5×10^4 cells ml^{-1} (250 μl per well in 48-well

plates. Sparse cell seeding was maintained throughout this study). After one day, the growth media was changed to CS 0.2% DMEM and the cells were incubated for 12-18 h. Cells were then treated with the assay samples for 30 min, followed by stimulation with 30 ng ml^{-1} of EGF (Sigma) for 30 min and observed under microscopy.

For screening, isolation from the broth, and evaluation of compounds, cells with complete absence of filopodia were judged to be filopodia inhibited. To quantify the filopodia cell population, filopodia protrusion was induced in the same manner as above except that cells were seeded on glass cover slips in 12-well plates. The cells formed colonies on the cover slips, and filopodia cell colonies were then counted. Colony counts were done in nine fields chosen at random for one sample.

Cellular ATP levels were determined using an ATP assay kit (Sigma) after cells had been treated for 30 min treatment with test compounds.

For the test of inhibitors of mitochondrial respiration, concentrations of 100 nM rotenone, 10 ng ml^{-1} of antimycin A and 10 ng ml^{-1} of oligomycins were

used.

CE-TOFMS metabolomics

Cells grown in 100-mm dishes were incubated in serum-reduced media for 18 h, and then treated with test compounds for 30 min. After washing cells twice with ice-cold 5% mannitol, metabolites were extracted by keeping cells resting on ice for 10 min in 1 ml of ice-cold methanol containing internal standards (25 μM each of 3-aminopyrrolidine (Aldrich), L-methionine sulfone (Wako), trimesate (Wako), and MES (Wako)). Extracts were then transferred to a separate tube and mixed with 500 μl of milli-Q water, and 600 μl of this solution was transferred into another tube, mixed with 400 μl of chloroform, and centrifuged. A 300-μl aliquot of the aqueous layer was centrifugally filtered through a 5 kDa-cutoff membrane (Millipore) to remove proteins from samples. The filtrate was lyophilized, dissolved in 50 μl of milli-Q water, and subjected to CE-TOFMS analysis.

For the [^{13}C]-isotope labeling study, culture media was changed to glucose-depleted DMEM after 18 h incubation in serum-reduced media, and

cells were treated with 1 mg ml^{-1} of [1,2,3,4,5,6-^{13}C]-glucose (Isotec) immediately after the test compounds addition. Metabolites were extracted after 30 min. For the clear measurement of glucose-6-phosphate, fructose-6-phosphate, and glucose-1-phosphate, LC-MS systems were also used. Details of the metabolomic analysis are discussed in **Supplemental Experimental Procedures**.

***In vitro* hexokinase assay**

Hexokinase is very active when the enzyme is bound to mitochondria (Floridi, et al., 1981); therefore, hexokinase was isolated from crude mitochondria as described by Floridi *et al.*, with minor modifications.

Small fragments of bovine heart in MSH buffer (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 1 mM DTT, 0.1% BSA and 10 mM Hepes, pH 7.4) were homogenized with a Dounce Tissue Grinder and centrifuged at 1,000 × g for 10 min. The supernatant was further centrifuged at 8,000 × g for 20 min. The resulting pellet was homogenized in MSH buffer and centrifuged again. The crude mitochondria pellet was homogenized and stored at –80 °C until used.

For the measurement of hexokinase activity, G6PDH (Sigma) was used to generate NADPH from the product of the hexokinase reaction. Hexokinase activity was spectrophotometrically determined from the absorbance of NADPH at 340 nm (Bergmeyer, 1963). The reaction mixture contained 30 μg of bovine heart mitochondria, 1 mM ATP, 0.5 mM $NADP^+$, 2 μM rotenone (Calbiochem), 3 μg ml^{-1} oligomycins (Calbiochem), and 0.1 units of G6PDH in 100 μl of PT buffer (10 mM MOPS, 200 mM sucrose, 5 mM succinate, 1 mM P*i*, and 0.01 mM EGTA, pH 7.4). The reaction was initiated by addition of 0.2 mM glucose at room temperature after a 10-min pre-incubation period. The level of NADPH was continuously recorded for 20 min.

Uptake of [^{3}H]-2-deoxyglucose

Serum-starved A431 cells in DMEM containing 1.2 mM glucose were treated with test compounds along with 0.5 μCi of [1,2-^{3}H]-2-deoxy-D-glucose ([^{3}H]-2DG, specific activity 50-60 Ci $mmol^{-1}$, ARC) for 30 min, washed twice with ice-cold PBS, and lysed with 0.5 N NaOH. Cell lysate radioactivity was measured on a liquid scintillation counter.

Swiss 3T3-L1 pre-adipocytes were differentiated into adipocytes as described (Saito, et al., 2007). Adipocytes were pretreated with or without insulin (100 nM, Sigma) for 15 min for GLUT4 translocation, and then treated with test compounds and [^{3}H]-2DG for 5 min for the glucose uptake study. Other conditions were the same as described above.

To test GPA-sensitivity to GLUT1 overexpression, HEK293T cells were transfected with glut1 (derived from A431 cells) using Lipofectamine. In the uptake study, an HEK293T cell suspension was used because the cells are easily detached from the culture plates and it was quite difficult to wash cells immediately to terminate the uptake reaction. After 36 h from transfection, cells were left in PBS containing 1 mM EDTA for the gentle detachment. A suspension of 2.0×10^5 cells in 200 μl of glucose-free DMEM in a tube was treated with test compounds and [^{3}H]-2DG in a 25°C water bath for 5 min. Glucose uptake was terminated by the addition of ice-cold high glucose solution (final 25 mM), followed by centrifugation at 1,000 × g at 4°C for 5 min. Cell pellets were washed once with ice-cold high glucose solution and lysed with 0.1 N NaOH. [^{3}H]-2DG

uptake under this condition linearly increased for at least 30 min.

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M.I., T.S. and M.K. designed the study, analyzed the data, and wrote the paper. M.K. performed the experiments and analyzed the data, while S.I. performed the metabolome measurements. T.S. performed the metabolomic analysis, and E.T. and T.S. provided critical advice and contributed to writing the paper.

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Figure Legends

Figure 1. Synergistic inhibition of filopodia protrusion by co-treatment with GPA and PA.

(**A**) Planar structures of GPA and PA.

(**B**) Synergistic inhibitory activity of GPA & PA in combination against filopodia protrusion in A431 cells. Cells were treated with varied concentrations of PA and GPA for 30 min, then stimulated by EGF (30 ng ml^{-1}) for 30 min to protrude filopodia (as shown by arrowhead in control picture above). Frameless photos indicate cells with filopodia, while the framed photos indicate cells in which filopodia protrusion was inhibited. Note that co-treatment with GPA & PA inhibited filopodia protrusion while single treatment with each did not, indicating that GPA and PA act synergistically. Photos represent the results of three independent experiments.

(**C**) Quantification of inhibition of filopodia protrusion by GPA and PA co-treatment. The number of A431 colonies with filopodia were determined microscopically, and the rate of inhibition [filopodia colonies] / [total colonies] in a

field was calculated. Error bars represent ± standard deviation (n = 9). The same result was obtained in duplicate.

See also **Fig S1** and **Table S1** for the supporting data on the bioassay-guided isolation, the structural identification and the bioactivity of GPA & PA.

Figure 2. Mechanistic insights into synergistic inhibition of filopodia protrusion by GPA and PA.

(**A**) Synergistic inhibition of filopodia protrusion by inhibitors of mitochondrial respiration in the presence of GPA. Mitochondrial inhibitors with similar activity to PA also inhibited filopodia protrusion in the presence of GPA. Mitochondrial inhibitors RTN (rotenone: another inhibitor of complex I), AMA (antimycin A: complex III inhibitor), and OM (oligomycins: complex V inhibitor) were used.

(**B**, **C**) GPA appears to suppress glycolysis. Through chemical genomic screening of target-identified inhibitors, we found that the hexokinase inhibitor 2DG alone synergistically inhibited filopodia protrusion in the presence PA, similar to the effect observed with GPA (for (**B**), the entire screening results are

shown in **Table S2**). Since hexokinase inhibition by 2DG would suppress glycolysis, this suggested that glycolytic suppression might be responsible for the synergistic inhibition of filopodia protrusion with PA, and that therefore GPA might also suppress glycolysis. This validity of this hypothesis was supported by the result demonstrating that GPA decreases cellular ATP in the presence of the mitochondrial respiration inhibitor PA (**C**), since it is known that glycolytic suppression causes a drastic decrease in cellular ATP under the suppression of mitochondrial respiration. Error bars: standard deviation (n = 3).

The respiratory inhibition by PA and the weak inhibition by GPA were in **Fig S2**.

Figure 3. Snapshot picture of GPA-changed metabolome.

The global metabolites in GPA-treated A431 cells (30 min treatment) were extracted and analyzed by CE-TOFMS, and compared with that of control cells. Significantly decreased metabolites in GPA-treated cells are shown as ▩, ▩, and ■ (fold decrease > 50%, > 25%, and < 25%, respectively), and significantly increased metabolites are shown as □, ▩, and ■ (fold increase

< 200%, < 400%, and > 400%, respectively). Significance was determined by Student's *t*-test (n = 4, $p < 0.05$). The entire set of results with exact *p* values and list of metabolite abbreviations are listed in **Table S3**. (See also **Fig S3** for lactate / pyruvate ratio in addition)

Figure 4. [^{13}C]-labeling study.

[^{13}C]-glucose was incorporated into A431 cells for 30 min immediately after GPA-treatment. All detected major isotopomers ([^{13}C]-glucose-6-phosphate and its [^{13}C]-metabolites) were decreased by GPA-treatment (the entire list is shown in **Table S4**), indicating that GPA inhibits the step from [^{13}C]-glucose uptake to [^{13}C]-glucose-6-phosphate production. Error bars: standard deviation (n = 4).

Figure 5. GPA-mediated inhibition of glucose uptake via GLUT.

(**A**) GPA failed to inhibit the *in vitro* hexokinase enzyme reaction. Previously, hexokinase activity was successfully monitored by addition of the hexokinase substrate glucose, causing an increase in enzymatic activity (detected by