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Metabolomic Identification of the Target of the Filopodia Protrusion Inhibitor Glucopiericidin A

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SUMMARY

Identifying the targets of bioactive compounds is a major challenge in chemical biological research. Here, we identified the functional target of the natural bioactive compound glucopiericidin A (GPA) through metabolomic analysis. We isolated GPA while screening microbial samples for a filopodia protrusion inhibitor. Interestingly, GPA alone did not inhibit filopodia protrusion, but synergistically inhibit protrusion with the mitochondrial respiration inhibitor, piericidin A (PA). These results suggested that GPA might inhibit glycolysis. Capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) provided strong evidence that GPA suppresses glycolysis by functionally targeting the glucose transporter. GPA may therefore serve as a glucose transporter chemical probe. Simultaneous inhibition of both glycolysis and mitochondrial respiration dramatically decreased intracellular ATP levels, indicating that GPA inhibits ATP-dependent filopodia protrusion with PA. Our results represent a challenge of molecular target identification using metabolomic analysis.

INTRODUCTION

To fully understand the regulation of cellular events (e.g., cell growth, survival, migration), functional analysis of each protein involved in the regulatory systems is required (Pandey and Mann, 2000). Genetic studies utilizing chemical inhibitors are among the best approaches to investigate protein function (Alaimo et al., 2001; Zheng and Chan, 2002). Chemical inhibitors can be used to modulate the function of target proteins in a manner analogous to the engineering of mutations in molecular genetic studies (Alaimo et al., 2001; Fenteany et al., 1995; Liu et al., 1991). Many chemical inhibitors are available, though there are far fewer than the number of proteins involved in the complicated regulation of cellular events. Therefore, new chemical inhibitors must be developed in order to advance the applicability of chemical genetic studies to the functional analysis of proteins.

The structural diversity of natural products makes them ideal screening sources for chemical inhibitors that can be used to dissect the complex molecular mechanisms underlying cellular events through chemical genetics (Abel et al., 2002; Newman and Cragg, 2007). One strategy for screening natural products for new chemical inhibitors is the cell-based assay (Hart, 2005). However, identifying the target of a molecular inhibitor isolated by cell-based assays represents a crucial hurdle that must be overcome before chemical genetic studies can commence.

There are two fundamental approaches to identify chemical inhibitor targets: direct and indirect (Hart, 2005). In the direct approach, the target proteins bound to the inhibitor are purified and directly identified by mass spectrometry. In this approach, chemical synthesis of the inhibitor compound onto immobilized beads or a column is required for affinity purification of the binding proteins. However, in some cases, the chiral centers and unique structural scaffolds of natural products make synthesis difficult.

The indirect approach to chemical inhibitor target identification involves searching for candidates by profiling biological data. If the compound was found to perturb some cellular event for which the regulatory signaling pathway is known, targets can be revealed by examining the effect of the compound on each step of the pathway. In some cases, omics studies (e.g., proteomics, transcriptomics, metabolomics) can aid the comprehensive investigation of a compound's effect on the potentially large numbers of biological steps (Hart, 2005).

We employed an indirect approach, using metabolomics to identify the chemical inhibitor derived from natural product screening. Metabolomics technologies have advanced tremendously in recent years, and capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) has emerged as a powerful new tool for the comprehensive analysis of cellular metabolites (Monton and Soga, 2007; Soga et al., 2003). The use of CE-TOFMS to understand global metabolism at the system level has become widespread (Hirayama et al., 2009; Ishii et al., 2007; Soga et al., 2006; Sugimoto et al., 2009). Analysis of the metabolome with CE-TOFMS also revealed metabolic changes induced by drug compounds (Soga et al., 2006). Thus, despite a lack of reports describing the identification of chemical

Table 1. Inhibitory Activities of Remixing Silica Gel Chromatography Fractions, and the Effect of Withholding Each Fraction from the Remix

	Fractions Eluted with $CHCl_3$: MeOH						
	100: 0	100: 1	100: 2	100: 5	100: 10	100: 30	
Sample #	I	II	III	IV	V	VI	Filopodia Protrusion
Remix	Contained	Contained	Contained	Contained	Contained	Contained	Inhibition
Remix – **I**		Contained	Contained	Contained	Contained	Contained	**No inhibition**
Remix – **II**	Contained		Contained	Contained	Contained	Contained	Inhibition
Remix – **III**	Contained	Contained		Contained	Contained	Contained	Inhibition
Remix – **IV**	Contained	Contained	Contained		Contained	Contained	**No inhibition**
Remix – **V**	Contained	Contained	Contained	Contained		Contained	Inhibition
Remix – **VI**	Contained	Contained	Contained	Contained	Contained		Inhibition
I + **IV**	Contained			Contained			**Inhibition**
I	Contained						No inhibition
IV				Contained			No inhibition

See Figure S1 for the bioassay-guided isolation of the compounds.

inhibitor targets using metabolomic analysis, such efforts would be worthwhile.

We began our investigation by conducting a natural product screening to isolate bioactive compounds that inhibit cellular filopodia protrusion in the carcinoma. Filopodia are spike-like cell membrane projections contributing to tumor metastasis; however, the molecular mechanisms controlling filopodia protrusion are complicated and unclear (Faix and Rottner, 2006; Mattila and Lappalainen, 2008). Finding a filopodia inhibitor in carcinoma and its molecular target that could be employed in chemical genetic studies may therefore lead to a fuller understanding of filopodia contributing to the treatment of tumor metastasis (Bacon et al., 2007; Shulman et al., 2009). Here, we describe the isolation of a new chemical inhibitor of filopodia protrusion through natural product screening, and identification of the inhibitor's target by metabolomic analysis. This is the first report to our knowledge on natural product target molecule identification using a metabolomic approach.

RESULTS

Screening and Isolation of the Inhibitor of Filopodia Protrusion

Human epidermal carcinoma A431 cells highly express the epidermal growth factor (EGF) receptor. In response to EGF stimulation, these cells become highly chemotactic (Rabinovitz et al., 1999). Within 30 min of EGF stimulation, A431 cells protrude large spike-like filopodia that can be easily observed under a microscope. We used this simple assay system to identify compounds from microbial sources that inhibited filopodia protrusion.

After screening over 3000 microbial broth samples, we found that one broth from *Lechevalieria sp.* strain 1869-19 strongly inhibited EGF-induced filopodia protrusion in A431 cells (see Figure S1A available online).

To isolate the potential inhibitors in this broth, the broth extracts was separated into six fractions, numbered **I**–**VI**, by silica-gel chromatography with chloroform-methanol elution. However, no single fraction showed inhibitory activity against filopodia protrusion (Figure S1B). To examine whether the active substance may have degraded during chromatography, all six fractions were remixed (recombined fractions were termed "Remix") and tested for inhibitory activity. The Remix inhibited EGF-induced filopodia protrusion in the same concentration range as the broth extract (Figure S1C). These results suggested that the inhibition of EGF-induced filopodia protrusion was due to the synergistic action of two (or more) substances in the broth extract, and that these "active" substances were separated into different fractions during silica gel chromatography.

To determine which of the six fractions contained each active substance, we prepared remixes with one fraction withdrawn and assessed each such remix for ability or inability to inhibit filopodia protrusion. Withdrawal of fractions **I** (eluted from silica gel column by 100:0 chloroform:methanol) and **IV** (eluted by 100:5 chloroform:methanol) from the Remix resulted in no inhibition of filopodia protrusion (Table 1; Figure S1D), indicating that fractions **I** and **IV** contained active substances. Furthermore, combining fractions **I** and **IV** restored the inhibitory activity to a level comparable to the complete Remix, indicating that the mixture of the active substances in fractions **I** and **IV** was sufficient for inhibition of EGF-induced filopodia protrusion.

To identify the active substances in fractions **I** and **IV**, further isolation processes were undertaken. Fraction **IV** was subjected to silica gel column chromatography with toluene-acetone elution. Each resulting fraction was assessed for its ability to inhibit filopodia protrusion in the presence of fraction **I**. Fractions eluted with a toluene-acetone mixture at a 3:1 ratio demonstrated inhibition in the presence of fraction **I**, but not in its absence. Fractions showing the filopodia inhibition were collected and further purified by centrifugal liquid-liquid partition chromatography (CPC) using chloroform-methanol-water (5:4:6, ascending mode) elution, yielding a colorless, amorphous product. Analysis of MS and NMR spectra identified the product as glucopiericidin A (GPA) (Matsumoto et al., 1987) (Figure 1A).

The active substance in fraction **I** was purified by CPC using hexane-ethyl acetate-acetonitrile (5:1:4, ascending mode) elution, yielding pure yellow oil. This product was identified by MS

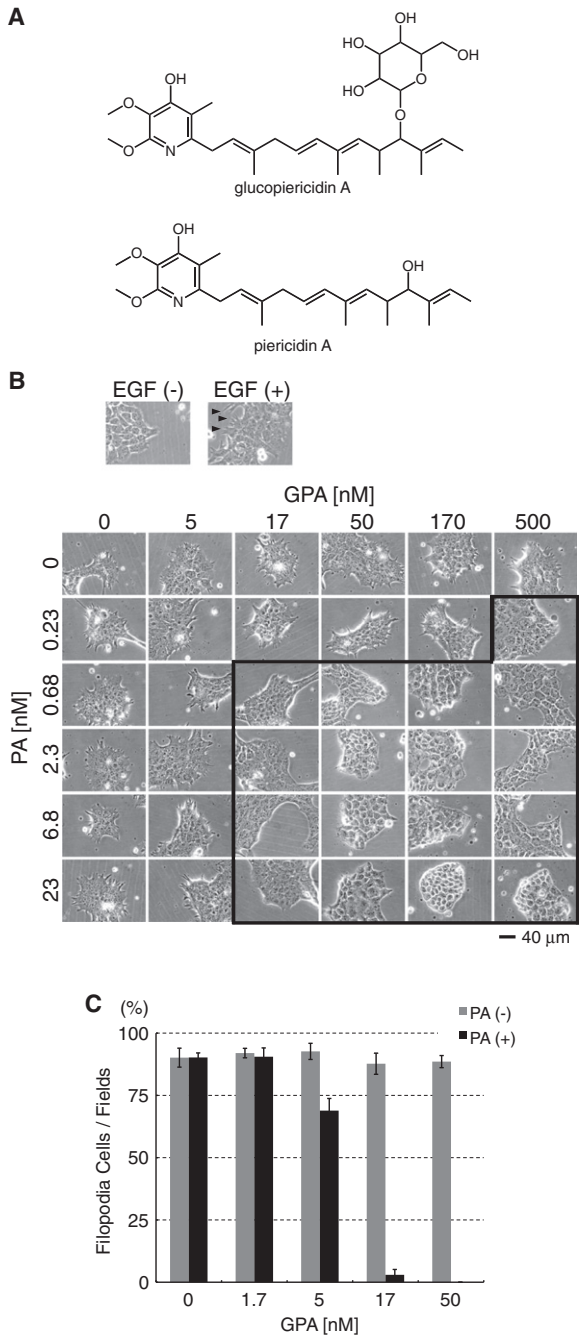

Figure 1. Synergistic Inhibition of Filopodia Protrusion by Cotreatment with GPA and PA

(A) Planar structures of GPA and PA.

(B) Synergistic inhibitory activity of GPA and PA in combination against filopodia protrusion in A431 cells. Cells were treated with varied concentrations of PA and GPA for 30 min and then stimulated by EGF (30 ng ml^{-1}) for 30 min to protrude filopodia (as shown by arrowheads in control picture above). Frameless photos indicate cells with filopodia, while the framed photos indicate cells in which filopodia protrusion was inhibited. Note that cotreatment with GPA and 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 cotreatment. 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 ± SD (n = 9). The same result was obtained in duplicate.

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

and NMR as piericidin A (PA) (Hall et al., 1966) (Figure 1A). The final purification procedure is described in Figure S1E.

Next, we examined the inhibitory activity of GPA and PA against filopodia protrusion (Figures 1B and 1C). Neither GPA nor PA alone, at concentrations up to 500 nM and 2.3 μM, respectively, showed inhibitory activity (see also Figure S1F). When combined, however, much lower concentrations of GPA (17 nM) and PA (0.68 nM) produced inhibition of filopodia protrusion. Hence, we isolated two natural products that inhibit EGF-induced filopodia protrusion in a synergistic manner.

PA-Mediated Inhibition of Mitochondrial Respiration Is Necessary for Inhibition of Filopodia Protrusion

Next, we investigated the mechanisms underlying the synergistic inhibition of filopodia protrusion using cotreatment with GPA and PA. Since PA is a known inhibitor of the mitochondrial respiratory chain complex I (Gutman et al., 1970; Hall et al., 1966), we hypothesized that inhibition of mitochondrial respiration by PA was required for the observed synergistic inhibition of filopodia protrusions when cells were cotreated with GPA and PA. To test this hypothesis, we examined whether other inhibitors of mitochondrial respiration would inhibit filopodia protrusion in combination with GPA. As expected, rotenone, another complex I inhibitor, showed the synergistic inhibition in combination with GPA (Figure 2A). In addition, antimycin A, a mitochondrial respiratory chain complex III inhibitor, and oligomycins, complex V inhibitors, also inhibited filopodia protrusion synergistically with GPA (Figure 2A). These results suggested that suppression of mitochondrial respiration is responsible for the synergistic filopodia inhibition seen with GPA and PA. Conversely, although GPA is the glucopyranoside derivative of PA, GPA showed low inhibitory activity against mitochondrial respiration (about 500-fold weaker than PA) (see Figure S2). The weak inhibition of mitochondrial respiration suggested that GPA must contribute to inhibition of filopodia protrusion through a different mode of action.

Elucidating GPA's Mode of Action through CE-MS Metabolomics

We conducted chemical genomic screening to investigate the mode of action whereby GPA contributes to the inhibition of filopodia protrusion. In this screening, the biological profile of target-identified inhibitors of filopodia inhibition was compared with that of GPA in order to find target-identified inhibitors with the same bioactivity as GPA, considering the possibility that GPA might target the same molecules as these other inhibitors. About 200 target-identified compounds were assessed for their ability to inhibit EGF-induced filopodia protrusion in the presence of PA (Table S2). As a result of this analysis, we found that 2-deoxyglucose (2DG), a known suppressor of glycolysis (Bachelard et al., 1971), was the sole compound able to inhibit protrusion in combination with PA (Figure 2B).

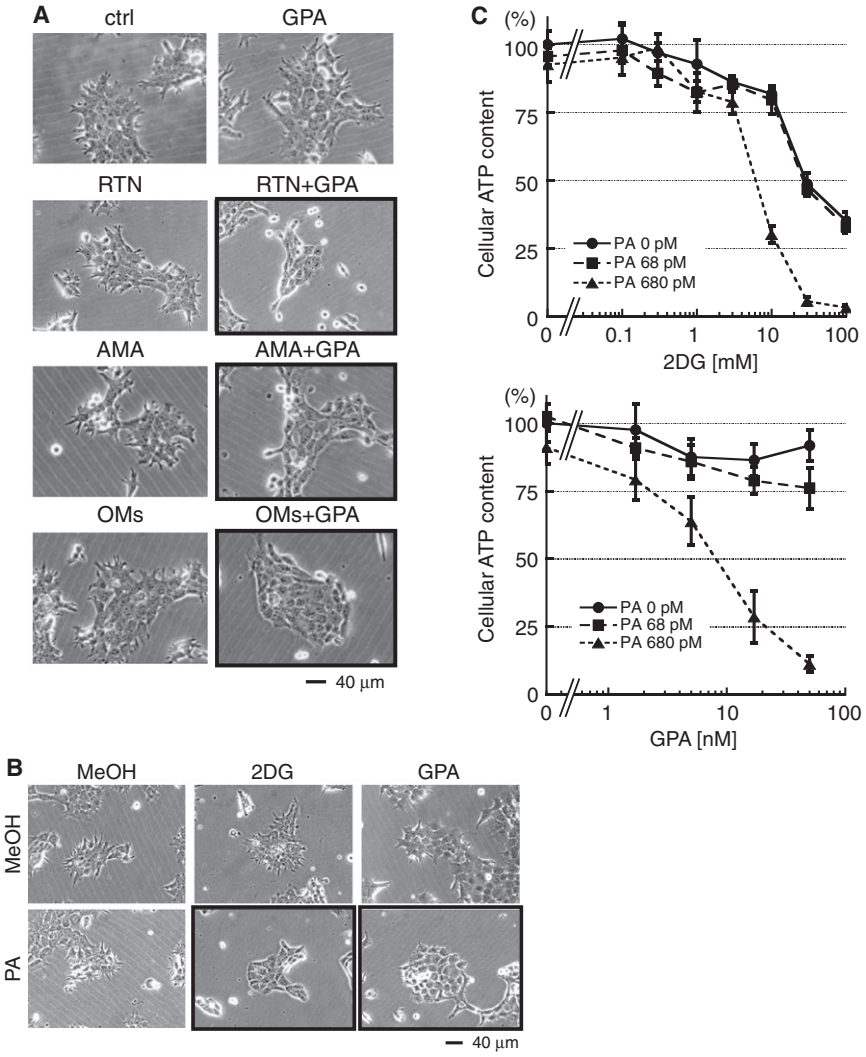

Figure 2. Mechanistic Insights into Synergistic Inhibition of Filopodia Protrusion by GPA and PA

(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 and 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: SD (n = 3).

The respiratory inhibition by PA and the weak inhibition by GPA are shown in Figure S2.

This raised the possibility that glycolytic suppression could be involved in the synergistic inhibition of filopodia protrusion seen with PA. Additionally, filopodia protrusion has been described as an ATP-dependent process (Le Clainche and Carlier, 2008). Thus, we hypothesized that PA and 2DG inhibit filopodia protrusion by decreasing cellular ATP levels through simultaneous blockage of two ATP-producing metabolic pathways: glycolysis (2DG) and mitochondrial respiration (PA). In support of this hypothesis, we found that 2DG did indeed decrease cellular ATP levels synergistically with PA (Figure 2C). We therefore suspected that GPA might also decrease cellular ATP levels, resulting in inhibition of filopodia protrusion in the same manner as 2DG. As shown in Figure 2C, treatment of A431 cells with GPA also caused a drastic decrease in cellular ATP with PA, suggesting that GPA might perturb the ATP-producing metabolic pathways, most likely glycolysis, resulting in the filopodia protrusion inhibition in the presence of PA.

One possible approach to address the issue of whether GPA actually perturbs glycolysis is to assess the effect of GPA on global metabolism and on glycolysis in particular, by measuring the metabolome using CE-TOFMS. In CE-TOFMS, metabolites are separated by capillary electrophoresis (CE) on the basis of their charge and size, and then detected as peaks in MS (Soga et al., 2003). Metabolites are identified by comparing CE migration time and molecular weight with that of standards, and are quantified by peak intensity in reference to standard calibration curves. We used this approach to quantitatively examine the differences in metabolite levels between control and GPA-treated cells.

Using the CE-TOFMS approach, we detected around 4000 (3853–4848) peaks in each sample (control and GPA-treated, n = 4). A reference set of 112 standards was used to identify the metabolites of glycolysis and related pathways (pentose phosphate pathway, TCA cycle, nucleotide synthesis, amino acid, and others, see Figure 3). A total of 83 peaks were quantitatively identified, and 24 metabolites were found to significantly differ between control and GPA-treated samples (for the entire list with exact *p* values, see Table S3), which are mapped in the overview in Figure 3 (pathway information was obtained by reference to KEGG [http://www.genome.jp/kegg/pathway.html]).

Our results showed that GPA significantly decreased the amount of glycolytic end products (pyruvate: 61.5%, $p = 3.8 \times 10^{-5}$ and lactate: 40.3%, $p = 1.4 \times 10^{-5}$). Moreover, another glycolytic parameter, the lactate/pyruvate ratio, was also

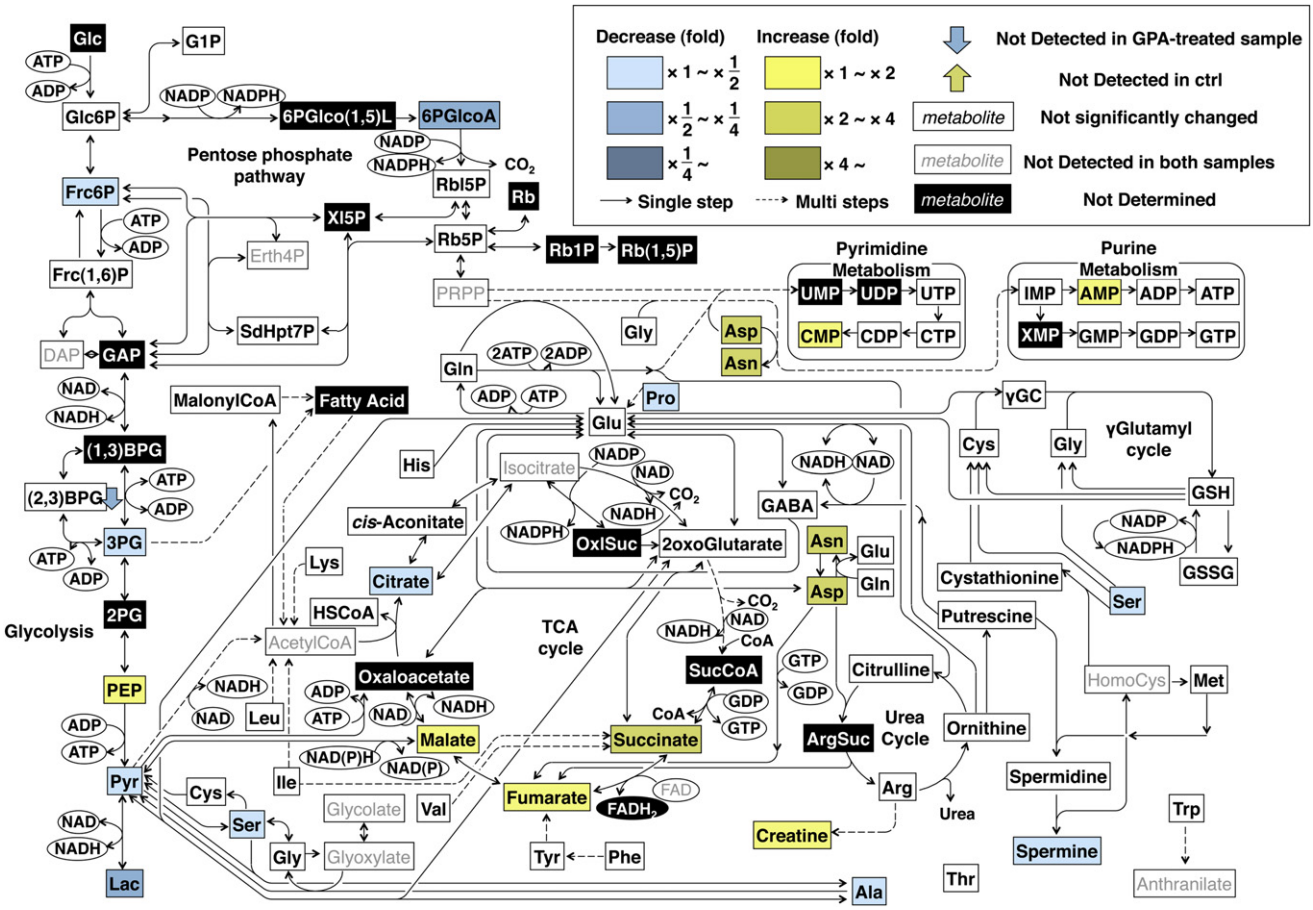

Figure 3. Snapshot Picture of GPA-Changed Metabolome

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 Figure S3 for lactate/pyruvate ratio.)

significantly decreased (Figure S3). These results strongly suggested that GPA-inhibited glycolysis.

GPA Targets Glucose Uptake

Upon determining that GPA inhibits glycolysis, we then examined which reaction step in glycolysis was blocked by GPA as a means of identifying the GPA target. To reveal particular GPA-induced changes in glucose metabolism, control and GPA-treated A431 cells were treated with [1,2,3,4,5,6-^{13}C]-glucose (full-label), and then the [^{13}C]-incorporated metabolites within those cells were quantified by CE-MS. Incorporation of [^{13}C]-labeled glucose into any metabolites of glycolysis was decreased in GPA-treated cells (Figure 4; Table S4), indicating that the glycolytic influx was lowered by GPA treatment. Since the metabolite closest to glucose in the glycolytic pathway is glucose-6-phosphate (G6P), these results suggest that GPA acts on the steps proceeding from glucose uptake to G6P production.

Since hexokinase catalyzes G6P production, we examined the effect of GPA on hexokinase activity in vitro. Glucose, as a substrate of hexokinase, was mixed with reaction buffer containing partially purified hexokinase, G6PDH, and NADP as a coenzyme of the G6PDH reaction, and incubated at room temperature (Bergmeyer, 1963; Floridi et al., 1981). In this reaction, the G6P produced by hexokinase is further hydrolyzed by G6PDH, with the accompanying conversion of NADP to NADPH, so that hexokinase activity can be estimated by the spectrophotometric absorbance of NADPH (Bergmeyer, 1963). As shown in Figure 5A, the assay revealed that addition of glucose led to an increase in the amount of NADPH, which could be negated by the hexokinase inhibitor 2DG. However, GPA at concentrations up to 1.7 μM failed to inhibit the reaction, indicating that the hexokinase reaction step is not the point of inhibition of glycolysis through a decrease in the amount of G6P by GPA.

Another possible step that GPA could act upon to cause a decrease in G6P is glucose uptake. To examine this possibility, we treated cells with [^{3}H]-2DG and evaluated whether GPA would affect glucose uptake. The compound 2DG acts as a glucose mimic and is incorporated into cells by glucose transporters. However, 2DG cannot be catabolized in glycolysis; thus, the amount of 2DG that is taken up simply reflects the glucose uptake capacity of a cell's glucose transporters.

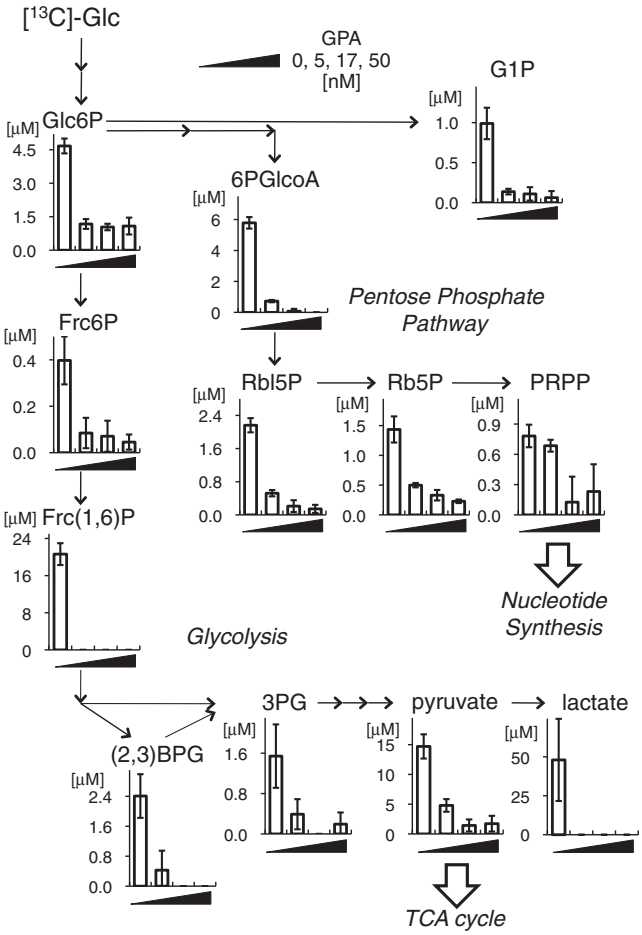

Figure 4. [^{13}C]-Labeling Study

[^{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: SD (n = 4).

In our experiments, GPA inhibited the uptake of [^{3}H]-2DG in a dose dependent manner (Figure 5B), with an IC_{50} of 4.9 ± 2.9 nM (mean ± SE, n = 3). This concentration was quite similar to the concentration of GPA required to inhibit EGF-induced filopodia protrusion in combination with PA (Figures 1B and 1C). It is reported that GLUT1 is responsible for glucose uptake in A431 cells (Aloj et al., 1999). Treatment of cells with GPA did not change the expression level of GLUT1 in the plasma membrane (Figure S5A), which suggests that GPA inhibits GLUT1 function.

To confirm whether GPA does in fact inhibit GLUT1 function, GLUT1 was overexpressed in cells (Figure S5B), and the sensitivity of cells to GPA upon 2DG uptake was evaluated. Uptake of 2DG was substantially increased (about 4-fold) by GLUT1 overexpression (expression level was shown in Figure S5B). Although GPA could inhibit 2DG uptake in GLUT1-overexpressing cells, when compared with vector control cells, GLUT1-overexpressing cells became less sensitive to GPA depending on the expression level of GLUT1 (Figure 5C). The IC_{50} value for 2DG uptake increased with GLUT1 expression (21.6 ± 7.7 nM, [mean ± SE, n = 3) in vector control cells versus 108 ± 24 nM (mean ± SE, n = 3) in GLUT1-overexpressing cells). These results indicated that GPA inhibits GLUT1 function, and thereby inhibits glycolysis.

DISCUSSION

In this study, we conducted natural product screening to isolate the inhibitor of EGF-induced filopodia protrusion. Natural products have historically provided a variety of important bioactive compounds, such as antibiotics, immunosuppresants, and antitumor agents (Abel et al., 2002; Lokey, 2003; Newman and Cragg, 2007). Natural product screening has also led to discovery of unique bioactive compounds useful in chemical genetic research (e.g., FK506 [Liu et al., 1991] and lactacystin [Fenteany et al., 1995]). Natural products often contain unique structures and chiral centers, which anecdotal evidence suggests are important for the unique bioactivity and target recognition of many compounds. We therefore conducted natural product screening in microorganisms to isolate and characterize an inhibitor of filopodia protrusion.

As a result of our screening studies, we found that the cultured broth of *Lechevalieria sp.* strain 1869-19 contained compounds that inhibited EGF-induced filopodia protrusion (Figure 1A). However, this inhibition required the synergistic effect of two compounds (Figures S1D and S1E), which were isolated and identified as GPA and PA (Figures 1A–1C).

We then examined the mechanism underlying the synergistic inhibition of filopodia protrusion caused by GPA and PA. PA is a well-known inhibitor of respiratory complex I in mitochondria (Gutman et al., 1970). Because other mitochondrial respiratory inhibitors, such as antimycin A and oligomycins, inhibited filopodia protrusion only in the presence of GPA, inhibition of mitochondrial respiration by PA could be responsible for the synergistic inhibition of GPA and PA (Figure 2A). Little is known about GPA however. There are few reports in the literature that describe the biological activities of GPA (Ahn et al., 1995; Matsumoto et al., 1987), and its molecular target(s) and mode of action have not been determined. To address this issue, we employed a chemical genomic approach, in which a target-identified chemical library was searched to find compounds with activity similar to GPA. The compound 2-deoxy-glucose (2DG) was identified using this approach (Figure 2B; Table S2).

Because 2DG is known to inhibit glycolysis by inhibiting the enzyme hexokinase, GPA would be expected to also inhibit glycolysis. By means of metabolomic analysis using CE-TOFMS (Figure 3) in combination with an evaluation of [^{13}C]-labeled glycolytic metabolites (Figure 4), we clearly determined that GPA disrupts glycolysis by inhibiting the initial step of the pathway. Finally, we have also clarified that glucose transporters are functional target proteins of GPA (Figure 5).

Glucose transporters comprise a large family of transmembrane proteins characterized by having different affinities for glucose (Mueckler et al., 1985; Scheepers et al., 2004). Among glucose transporters, GLUT1 has a high affinity for glucose and is one of the most abundantly expressed transporters in many types of tumors (Scheepers et al., 2004). Indeed, A431 cells used in this study have been reported to express only

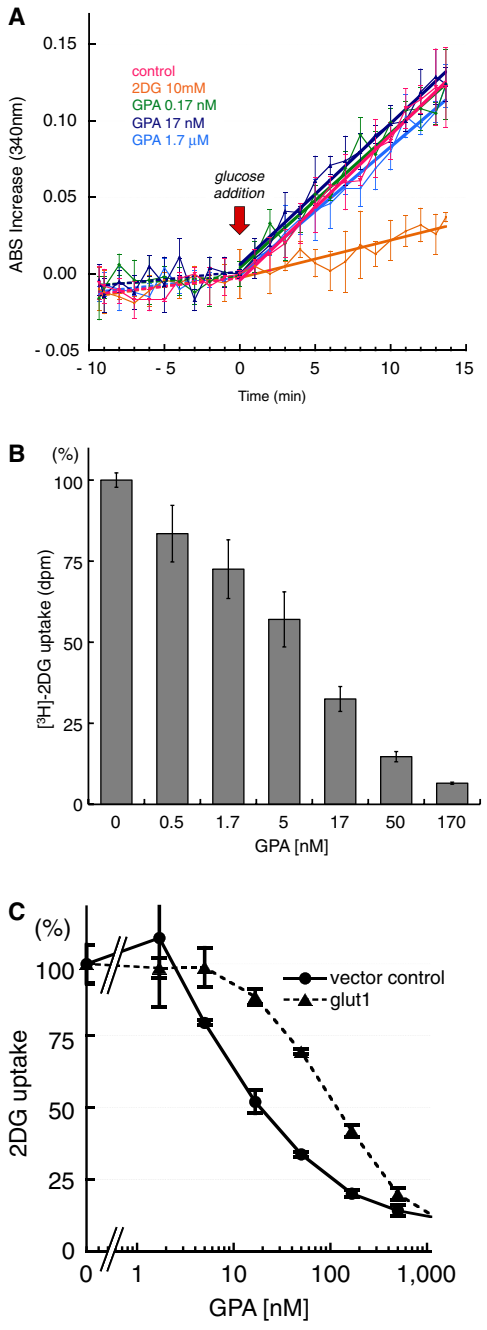

Figure 5. GPA-Mediated Inhibition of Glucose Uptake via GLUT

(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 NADPH absorbance at 340 nm), which was inhibited by 2-deoxyglucose. However, GPA could not inhibit this increase in hexokinase activity, indicating that GPA was not a hexokinase inhibitor. Error bars: SD (n = 3).

(B) Inhibition of glucose uptake by GPA. GPA inhibited the uptake of the glucose mimic 2-deoxyglucose (2DG) in a dose-dependent manner. Error bars: SD (n = 3). Similar results were obtained from three individual experiments. These results explain the glucose-6-phosphate decrease depicted in Figure 4.

(C) Inhibition of GLUT1-mediated glucose uptake by GPA. Since glucose uptake in A431 cells is primarily mediated by GLUT1, A431 GLUT1 was overexpressed, and the sensitivity of glucose uptake to GPA was examined. Overexpression lowered the sensitivity 2DG uptake inhibition by GPA as observed with the shift of the inhibition curve to the right. Error bars: SD (n = 3). The data shown represent results of three independent experiments.

(See also Figure S5.)

GLUT1 as glucose transporter (Aloj et al., 1999). Therefore, data accumulated in this study suggest that there are only three likely mechanisms that could explain glucose uptake inhibition by GPA: (i) GPA mimics the GLUT1 substrate, (ii) inhibition of GLUT1 translocation into the cell membrane, and (iii) lowering the expression level of GLUT1. We ruled out the last two possibilities by immunoblotting, as shown in Figure S5A. The data showing that the level of GLUT1 expression was not changed in the membrane fraction by GPA treatment suggest that uptake inhibition by GPA was not due to an inhibition of GLUT1 translocation or a reduction in GLUT1 expression. Moreover, GPA possesses the glucose moiety, and glucose null derivative PA failed to inhibit the uptake (Figure S5C). Thus, it is likely that the glucose uptake inhibition by GPA would be due to its glucopyranoside moiety, perhaps by functioning as a glucose mimic to GLUT1. This suggests that GLUT1 would be the bona fide target of GPA due to GPA's inhibition of glycolysis in A431 cells.

Our overexpression study confirmed GPA inhibits glucose uptake via GLUT1 in A431 cells, but we also tested GPA against another glucose transporter, GLUT4 in Swiss 3T3 cells. Differentiated Swiss 3T3-L1 adipocytes take up glucose basally via GLUT1. However, when 3T3-L1 adipocytes are stimulated with insulin, GLUT4 is translocated to the membrane (Saito et al., 2007), and both GLUT1 and GLUT4 transport glucose into the cells. GPA inhibited both the basal uptake of [^{3}H]-2DG via GLUT1 and the increased uptake of glucose in insulin-stimulated cells (Figure S5D), suggesting that GPA inhibits glucose uptake via both GLUT1 and GLUT4.

The glucose transporter inhibitors cytochalasin B and phloritin are known to suppress glucose uptake at μM and mM concentrations, respectively. GPA, on the other hand, inhibits glucose uptake in A431 cells, Swiss 3T3-L1 adipocytes, and GLUT1-overexpressed HEK293T cells at nanomolar concentrations (Figures 5B and 5C; Figure S5D). Thus, ours is the first report to our knowledge of a GLUT inhibitor capable of suppressing glucose uptake at nanomolar concentrations.

Aside from its influence on glycolysis, GPA treatment also leads to changes in the amounts of TCA cycle intermediates, as well as increases in levels of aspartate and asparagine (Figure 3; Table S3). We believe these changes may be caused in a part by GPA-limited entry of pyruvate into the citrate cycle, which might slow the rate at which oxaloacetate is converted to citrate. Slowing the conversion of oxaloacetate may lead to accumulation of preceding TCA cycle intermediates such as malate, fumarate, and succinate.

Reports suggest that active glutaminolysis takes place in the metabolome of tumor cells (Deberardinis et al., 2007), described as anaplerotic ATP energy fueling through "truncated" TCA metabolism (from 2-oxoglurate to malate) without pyruvate entry (Parlo and Coleman, 1984; Piva and McEvoy-Bowe, 1998). In tumor glutaminolysis, highly incorporated glutamine is converted into glutamate and then into 2-oxoglutarate via aspartate transaminase, with production of aspartate as a byproduct

(Piva and McEvoy-Bowe, 1998). Thus, the possibility should not be excluded that these changes in the amounts of TCA cycle intermediates and aspartate are caused by glutaminolysis in addition to GPA-suppressed pyruvate entry into the TCA cycle (Kovacević et al., 1987).

CE-MS metabolome analysis is a powerful technique that may help elucidate the targets of the compounds, in the case the compounds impact glycolysis. Glutaminolytic analyses, and labeling studies using [^{13}C]-glutamine in combination with CE-TOFMS—analogous to our studies of GPA's effect on glycolytic pathways—might clarify the target-unverified compounds that disturb glutaminolysis. Chemical metabolomic analyses should therefore be encouraged, not only because they may further understanding of the physiological and pathological effects of many natural products, but also because such studies 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 cotreatment 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 cotreatment 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 remixing 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, to our knowledge, 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 1 day, the growth media was changed to CS 0.2% DMEM and the cells were incubated for 12–18 hr. 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 coverslips in 12-well plates. The cells formed colonies on the coverslips, 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 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 hr 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 hr 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 1000 × g for 10 min. The supernatant was further centrifuged at 8000 × 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 preincubation 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 preadipocytes 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 hr 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 1000 × 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.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2010.06.017.

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