INTRODUCTION

Heat shock proteins (Hsp) including Hsp90 are highly conserved chaperones that stabilize and/or traffic proteins, refold misfolded proteins, and decide the fate of denatured proteins in cells (Pratt and Toft, 2003; Wandinger et al., 2008). Geldanamycin (GA) and its derivative, 17-allylamino-17-demethoxygeldanamycin (17-AAG), bind to the N-terminal ATPase domain of Hsp90, thereby inhibiting the ATP-dependent progression of the Hsp90 complex toward the stabilizing form and shifting it forward the proteasomal-targeting form, resulting in degradation of the Hsp90 client proteins. It has been established that many proteins including transcription factors and the proteins involved in cellular signaling such as receptors, Src kinases, and the Raf family, are Hsp90 clients, and treatment with Hsp90 inhibitors elicits various cellular responses including the expression of Hsp family proteins, impaired functioning of mitochondria, and modulation of signaling cascades in cells (Pratt and Toft, 2003; Wandinger et al., 2008). Thus, inhibition of Hsp90 has diverse, stimulatory and inhibitory, effects on cytotoxicity depending on factors such as cell types and stimuli. In neuronal cells including rat pheochromocytoma PC12 cells, treatment with Hsp90 inhibitors including GA has two effects on cell fate in general: causing cell death/apoptosis by itself and enhancement of cytotoxicity in cells treated with reactive oxygen species.
(ROS)-related stimuli and/or stress specifically at greater concentrations (from 10 nM to 10 μM), and showing neuroprotective effects at lower concentrations (nM order) (Sano, 2001; Lu et al., 2002, 2009; Kim et al., 2003; Salehi et al., 2006; Clark et al., 2009). However, the precise mechanism(s) for the dual effects of Hsp90 inhibition on cell fate has not been established, and the effects of Hsp90 inhibitors on orthovanadate, Na3VO4, -induced cytotoxicity have not been elucidated.

Ceramide is known as a major cytotoxic factor in various cells (Posse de Chaves, 2006). Treatment with 17-AAG caused cell death via production of ceramide in several cancer cells in the presence of another signal inhibitor (Walker et al., 2010), and treatment with GA enhanced autophagy in carcinoma cells treated with an inhibitor of sphingosine kinase (Beljanski et al., 2010). However, possible changes in ceramide metabolism have not been elucidated in Hsp90 inhibitor-treated PC12 cells. Arachidonic acid (AA) is another cytotoxic factor in various cells including PC12 cells (Doroshenko and Doroshenko, 2007; Kurosawa et al., 2009; Nakamura et al., 2012). Although AA is released by various pathways, a major enzyme responsible for the stimulus-induced release of AA is group IVA phospholipase A2 (also known as α-type cytosolic PLA2, cPLA2α). The activity of cPLA2α is regulated by its phosphorylation mediated by mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinase (ERK), in addition to Ca2+ (Hirabayashi et al., 2004). Many stimuli including receptor stimulation, Na3VO4, and ROS including hydrogen peroxide (H2O2) convey signals mainly through the MAPK/ERK kinase (MEK)-ERK pathway, and MEK activity is regulated by upstream enzymes, a family of MEK kinases including Raf. Some members of Raf and MEK, in addition to receptor for epidermal growth factor (EGF) are Hsp90 clients. Thus, treatment with Hsp90 inhibitors may regulate cytotoxicity via modulation of ceramide metabolism and AA release in Hsp90 inhibitor-treated PC12 cells. In the present study, we investigated 1) changes in cell fate, ceramide metabolism, and AA release in Hsp90 inhibitor-treated PC12 cells with and without Na3VO4 and H2O2, and 2) changes in cell fate and AA release in GA-treated human cervical carcinoma HeLa cells.

MATERIALS AND METHODS

Materials

GA was purchased from LKT Laboratories (St. Paul, MN, USA). 17-AAG, H2O2, and Na3VO4 were from Wako (Osaka, Japan). 4β-Phorbol 12-myristate 13-acetate (PMA, an activator of protein kinase C) and A23187 were from Sigma (St. Louis, MO, USA). EGF (human, recombinant) and sorafenib were from Funakoshi (Tokyo, Japan) and American Chemicals Custom (San Diego, CA, USA), respectively. GA and 17-AAG were used at concentrations reported previously (Sano, 2001; Salehi et al., 2006; Clark et al., 2009). The concentrations of other reagents were also the same as those in previous reports (Taniguchi et al., 2007; Kurosawa et al., 2009; Tada et al., 2010). GA, 17-AAG, PMA, and A23187 were dissolved in dimethyl sulfoxide. The reagents were diluted with the medium or the buffer before the experiments, and the final concentration of dimethyl sulfoxide in assays was less than 0.5%. The vehicles had no effect on cytotoxicity and the release of AA with or without the stimuli including Na3VO4.

Cell culture, and assays for cell morphology, cell detachment, and lactate dehydrogenase (LDH) leakage

PC12 cells and HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (Thermo Trace, Ltd., Nobel Park, Australia), 100 IU/ml penicillin, and 100 μg/ml streptomycin in collagen-coated dishes (Type IV, BD Biosciences, Bedford, MA, USA) and dishes (NunclonTM, Nunc A/S, Roskilde, Denmark), respectively. The morphological changes were observed by phase contrast microscopy, when the cells achieved 30 ~ 40% confluence. When the cells achieved 70 ~ 80% confluence (sub-confluent stage), they were used for assays of cell detachment, LDH leakage, ceramide metabolism, and AA release. PC12 cells at the indicated stage were cultured in serum-free medium (500 μl) for 6 hr (or the period indicated) with and without Hsp90 inhibitors. Then, a 20-μl solution of Na3VO4 and H2O2 was added to culture dishes, and the cells further cultured at 37°C for 18 hr. The effects of 1 mM and 5 mM Na3VO4 and 50 μM and 100 μM H2O2 were examined. Since serum-deprivation alone caused marked toxicity in PC12 cells (Shimma et al., 2003), we used the cells without washing, and the proportion of serum remaining in dishes was approximately 0.2%. Cell detachment was estimated by counting the cells attached to the dishes after culture. LDH leakage was determined according to the manufacturer’s instructions (Roche, Basal, Switzerland).

Measurement of ceramide metabolites

4-Nitrobenzo-2-oxa-1,3-diazole (NBD)-labeled C6-ceramide (NBD-ceramide, NBD-ceramide having a C6-acyl chain) was from Molecular Probes (Eugene, OR, USA). PC12 cells on plates were cultured for 16 hr with
medium containing 0.2% serum, 0.1% fatty acid-free albumin, 10 mM HEPES (pH 7.4), and Hsp90 inhibitors, and then labeled with 5 μM NBD-ceramide for 30 min at the last culture-stage. The labeled cells were washed twice with NBD-ceramide-free medium and further incubated for 1 hr with Na₃VO₄. Lipid extraction was performed using a chloroform/methanol solution followed by vigorous vortex mixing, and the lipids in the organic and aqueous phases were analyzed on a TLC silica gel 60 plate (#105724, Merck, German) using 1-butanol: acetic acid: water (3:1:1) as the mobile phase, as described (Tada et al., 2010) with minor modifications. The fluorescence was detected with LAS1000-Plus (Fuji-Film, Tokyo, Japan; 470 nm excitation and 515 nm emission).

**Assay for release of AA**
The release of AA from cells was determined as described previously (Kurosawa et al., 2009). [³H] AA (7.9 TBq/mmol) was purchased from Amersham (Buckinghamshire, UK). Briefly, cells on 24-well plates were labeled overnight with medium containing 0.2% serum, 0.1% albumin, [³H]AA, and GA. The radioactivity incorporated in the cells was not changed by GA treatment. The labeled and washed cells were stimulated with reagents such as 5 mM Na₃VO₄ and 100 μM H₂O₂ for the periods indicated. Other reagents tested were described in legend to Fig. 3 and the text. The ³H content of the supernatant was estimated, with data calculated as a percentage of all the radioactivity incorporated (20,000 ~ 40,000 dpm per well). The release of AA without stimuli was 2 ~ 4% of the total incorporated in the cells. For quantitative analyses, the release was normalized as a percentage of the respective control value.

**Data presentation**
Values for cell detachment, LDH leakage, and release of AA are the mean ± S.E.M. for the indicated number of independent experiments performed in duplicate. Values for ceramide metabolism are the mean ± S.D. for the indicated number of independent experiments. In the case of multiple comparisons, the significance of differences was determined using a one-way analysis of variance with Dunnett’s or Tukey’s test. For pair wise comparisons, Student’s two-tailed t-test was used. P values < 0.05 were considered to be significant.

**RESULTS**

**Effects of Hsp90 inhibitors on cell detachment and LDH leakage in PC12 cells**
Treatment with 2 μM GA did not cause marked morphological changes at 6 hr (data not shown) and 24 hr (Fig. 1A), and the treatment had no effect on cell detachment (Fig. 1B) and on LDH leakage, a marker of cytotoxicity (Fig. 1C), at 24 hr after the treatment. Treatment with 10 μM GA for 24 hr slightly but significantly caused cell detachment: the percentage of attached cells was 92.3 ± 3.1% (n = 6, P < 0.05). The reagent also caused LDH leakage: the value was variable and approximately 10 ~ 15%. Treatment with GA at 10 nM ~ 1 μM alone had no effect on cell detachment or LDH leakage. Although treatment with 17-AAG, an analog of GA, at 2 μM did not cause cell detachment and LDH leakage after 24 hr, the reagent at 5 μM and 10 μM caused cell detachment: approximately 70 ~ 90% and 40 ~ 60% of cells were attached, respectively (n = 3), depending on experiments.

Next, we investigated the effects of GA and 17-AAG in the presence of other stimuli. Tyrosine phosphatase inhibitors including Na₃VO₄ are reported to affect the survival of neuronal cells including PC12 cells (Zheng et al., 2000; Gerling et al., 2004). Stimulation with 1 mM Na₃VO₄ did not cause cell detachment (Fig. 1B), although 5 mM Na₃VO₄ caused a response: the proportion of attached cells was 75.1 ± 11.2% (n = 5, P < 0.05). In the 2 μM GA-treated cells, 1 mM Na₃VO₄ significantly caused cell detachment (Fig. 1B) and LDH leakage (Fig. 1C). Similar enhancement of 1 mM Na₃VO₄-induced cytotoxicity was observed in 2 μM 17-AAG-treated cells (data not shown). In the 10 μM GA-treated cells (92.3 ± 3.1% of cells attached), Na₃VO₄-induced cell detachment was significantly enhanced: the proportion of attached cells was 75.1 ± 11.2% (n = 5, P < 0.05) and 25.7 ± 8.1% (n = 5, P < 0.05), respectively. Stimulation with 50 μM H₂O₂ by itself caused cell detachment, and the response was significantly enhanced in the 2 μM GA-treated cells (Fig. 1B). The H₂O₂ treatment also caused marked LDH leakage, and the response did not appear to be enhanced by GA treatment. In 10 μM GA-treated cells, LDH leakage with 50 μM H₂O₂ was greater than 40%. The cell detachment and LDH leakage induced by 100 μM H₂O₂ alone were marked: only 10.3 ± 2.3% of cells were attached (n = 4, P < 0.05), and the leakage was over 40%, as described in our previous report (Shimma et al., 2003). Treatment with GA and 17-AAG at 500 nM and 1 μM had marginal stimulatory effects on the cytotoxicity of 1 mM Na₃VO₄, and the two inhibitors at 10 nM ~ 300 nM did not protect PC12 cells against Na₃VO₄ and H₂O₂ under our conditions (data not shown).

**Changes in NBD-ceramide metabolism in PC12 cells treated with Hsp90 inhibitors**
The NBD-ceramide used in this study is converted into...
NBD-sphingomyelin (NBD-SM), NBD-glucosylceramide (NBD-GlcCer), NBD-ceramide-1-phosphate (NBD-C1P), NBD-caproic acid (C6-fatty acid), and non-fluorescent sphingosine in cells by respective enzymes (Tada et al., 2010). First, we analyzed levels of NBD-ceramide metabolites in PC12 cells that were cultured with 5 μM NBD-ceramide for 30 min at 37°C and washed with the ceramide-free medium (Fig. 2, 0 hr). NBD-GlcCer and NBD-SM were detected in addition to NBD-ceramide. The amount of NBD-GlcCer formed in 30 min in PC12 cells treated with 2 μM 17-AAG for 16 hr was significantly less than that in the control cells (Exp. I in Table 1). The amount of neither NBD-ceramide nor NBD-SM was modified by 17-AAG treatment. The amount of NBD-caproic acid extracted in the aqueous phase was marginal, because the lipid appeared to be excluded to extracellular spaces during the labeling and/or washing (Tada et al., 2010). Next, NBD-ceramide metabolism was examined after 1 hr of incubation of the labeled cells. The amount of NBD-GlcCer decreased, and those of NBD-caproic acid increased (Exp. II in Table 1). 17-AAG treatment significantly inhibited the formation of NBD-GlcCer without changing the amounts of other metabolites. Although the amount of NBD-ceramide in 17-AAG-treated cells appeared to be greater than that in the control, the change was not significant. Treat-

Fig. 1. Effects of GA on cell morphology and on Na3VO4- and H2O2-induced cell detachment and LDH leakage in PC12 cells. (A) Cell morphology was observed at 24 hr after 2 μM GA treatment. Similar results were obtained three times. In (B) and (C), PC12 cells were treated with 2 μM GA for 6 hr, and then further cultured with the indicated reagents for 18 hr. Numbers of cells attached to dishes (B) and LDH leakage (C) for 24 hr were determined. Data are means ± S.E.M. for the indicated number of independent experiments performed in duplicate. *P < 0.05 and **P < 0.05, significantly different from the control and the value without GA, respectively.

NBD-sphingomyelin (NBD-SM), NBD-glucosylceramide (NBD-GlcCer), NBD-ceramide-1-phosphate (NBD-C1P), NBD-caproic acid (C6-fatty acid), and non-fluorescent sphingosine in cells by respective enzymes (Tada et al., 2010). First, we analyzed levels of NBD-ceramide metabolites in PC12 cells that were cultured with 5 μM NBD-ceramide for 30 min at 37°C and washed with the ceramide-free medium (Fig. 2, 0 hr). NBD-GlcCer and NBD-SM were detected in addition to NBD-ceramide. The amount of NBD-GlcCer formed in 30 min in PC12 cells treated with 2 μM 17-AAG for 16 hr was significantly less than that in the control cells (Exp. I in Table 1). The amount of neither NBD-ceramide nor NBD-SM was modified by 17-AAG treatment. The amount of NBD-caproic acid extracted in the aqueous phase was marginal, because the lipid appeared to be excluded to extra-cellular spaces during the labeling and/or washing (Tada et al., 2010). Next, NBD-ceramide metabolism was examined after 1 hr of incubation of the labeled cells. The amount of NBD-ceramide decreased, and those of NBD-GlcCer, NBD-SM, and NBD-caproic acid increased (Exp. II in Table 1). 17-AAG treatment significantly inhibited the formation of NBD-GlcCer without changing the amounts of other metabolites. Although the amount of NBD-ceramide in 17-AAG-treated cells appeared to be greater than that in the control, the change was not significant. Treat-

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ment with 2 μM GA also inhibited the formation of NBD-GlcCer (Exp. III). Addition of 17-AAG and GA at 2 μM in the NBD-ceramide labeling step (30 min) and in the assay step (1 hr, 37°C) did not change ceramide metabolism in the test periods (data not shown). Addition of 5 mM Na₃VO₄ slightly but significantly increased the formation of NBD-GlcCer in control PC12 cells (Exp. II), although the response was not significant in some cases because of wide variation (Exp. III). In PC12 cells treated with 17-AAG and GA, the Na₃VO₄-induced formation of NBD-GlcCer was decreased. Stimulation with 5 mM Na₃VO₄ increased NBD-caproic acid formation approximately 1.3 ~ 1.5-fold in PC12 cells. The effect of Na₃VO₄ on the formation of NBD-caproic acid was limited in the 17-AAG- and GA-treated cells. In Exp. III, the amounts of NBD-ceramide and NBD-SM were not changed by GA treatment with and without Na₃VO₄ (data not shown). The amounts of NBD-C₁P formed with and without Na₃VO₄ were below the limit of detection. The effect of 1 mM Na₃VO₄ on ceramide metabolism was not clear in the period of 1 hr treatment in PC12 cells, like in other cells (Tada et al., 2010).

### Table 1. Effects of Hsp90 inhibitors on NBD-ceramide metabolism

<table>
<thead>
<tr>
<th></th>
<th>Exp. I: Labeling for 30 min (n = 4, %)</th>
<th>Exp. II: Incubation for 1 hr after labeling (n = 4, fold)</th>
<th>Exp. III: Incubation for 1 hr after labeling (n = 3, fold)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>17-AAG-treated</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>17-AAG-treated</td>
<td>17-AAG-treated</td>
<td>GA-treated</td>
</tr>
<tr>
<td>NBD-ceramide</td>
<td>100%</td>
<td>99.0 ± 7.9 (33.9 ± 1.0)</td>
<td>0.73 ± 0.05</td>
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<tr>
<td></td>
<td>(34.4 ± 2.8)</td>
<td>(34.4 ± 2.8)</td>
<td>1.33 ± 0.19</td>
</tr>
<tr>
<td>NBD-GlcCer</td>
<td>100%</td>
<td>81.1 ± 6.7 (4.34 ± 0.86)</td>
<td>0.87 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>(5.33 ± 0.69)</td>
<td>(5.33 ± 0.69)</td>
<td>1.55 ± 0.21</td>
</tr>
<tr>
<td>NBD-SM</td>
<td>100%</td>
<td>95.3 ± 8.3 (5.40 ± 1.03)</td>
<td>1.16 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>(5.70 ± 0.94)</td>
<td>(5.70 ± 0.94)</td>
<td>1.16 ± 0.25</td>
</tr>
<tr>
<td>NBD-caproic acid</td>
<td>(&lt; 0.1)</td>
<td>(&lt; 0.1)</td>
<td>(&lt; 0.1)</td>
</tr>
<tr>
<td></td>
<td>(2.0 ± 0.31 pmol)</td>
<td>(2.40 ± 0.61)</td>
<td>(2.07 ± 0.28)</td>
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</table>

PC12 cells were treated with vehicle, 2 μM 17-AAG (Exps. I and II), or 2 μM GA (Exp. III) for 16 hr, and then labeled with 5 μM NBD-ceramide for 30 min. The labeled and washed cells were incubated with 5 mM Na₃VO₄ (V) or vehicle (−) for 1 hr, and the NBD-ceramide metabolites were analyzed as described in Fig. 2. NBD-ceramide metabolites were analyzed immediately after the labeling (Exp. I) and at 1 hr after incubation with Na₃VO₄ (Exps. II and III). In Exp. I, the amounts of NBD-ceramide metabolites were expressed as a percentage of each metabolite without 17-AAG treatment, and the data in parentheses were expressed as pmol/well. In Exps. II and III, the data were expressed as the fold-increase of each metabolite in control cells without Na₃VO₄. The data in parentheses were expressed as the fold-change of each metabolite in control cells before 1 hr incubation or as pmol/well. P < 0.05 and *P < 0.05, significantly different from the control values without Hsp90 inhibitors and without Na₃VO₄, respectively.
Effect of GA on release of AA

AA metabolism couples with cytotoxicity in various cells including PC12 cells, as described in the Introduction.

Fig. 2. Effect of 17-AAG treatment on NBD-ceramide metabolism. PC12 cells were treated with vehicle and 2 µM 17-AAG for 16 hr, and then labeled with 5 µM NBD-ceramide for 30 min. The lipids in the labeled and washed cells were extracted (0 hr), and divided into organic (A) and aqueous (B) phases. The lipids in the two phases were analyzed separately. In some cases, the cells were incubated with and without 5 mM Na$_3$VO$_4$ for an additional 1 hr, and then the lipids both in the cells and in the buffer were extracted. For quantitative analyses, indicated amounts of standard NBD-ceramide (0 ~ 4 pmol) were spotted in the upper area of the plate after the separation by TLC. Data are from a typical experiment, and the quantitative data are shown in Table 1.

Fig. 3. Inhibitory effect of GA treatment on release of AA in HeLa cells, but not PC12 cells. PC12 cells and HeLa cells were labeled with [3H]AA in the presence of 2 µM GA for 18 hr. (A) PC12 cells were stimulated with and without 5 mM Na$_3$VO$_4$ in the presence of 2 µM A23187 for 1 hr. The absolute amounts of AA released are given in the text. Data were expressed as percentages of the respective value without GA, and are means ± S.E.M. (B) HeLa cells were stimulated with vehicle, 10 nM PMA plus 0.1 µM A23187 (PMA/A23187), and 20 ng/ml EGF plus 0.1 µM A23187 (EGF/A23187) for 30 min. Data are normalized as percentages of AA release without stimuli from control cells, and are means ± S.E.M. a, b P < 0.05, significantly different from control values and the values without GA, respectively.
tion. In PC12 cells, the release of AA without stimuli for 1 hr was not changed by treatment with 2 μM GA for 18 hr: 1.3 ± 0.2% (% of total AA incorporated) and 1.5 ± 0.2% with and without GA, respectively (n = 3). Stimulation with 5 mM Na3VO4 by itself did not release AA, and GA treatment did not change the release for 1 hr: values were 1.7 ± 0.2% and 1.8 ± 0.2% with and without GA treatment, respectively. Previously, we showed that stimulation with Na3VO4 and H2O2 released AA via cPLA2α’s activation in the presence of a Ca2+ ionophore, A23187 (Taniguchi et al., 2006, 2007). Stimulation with 2 μM A23187 markedly released AA for 1 hr in PC12 cells: 1.3 ± 0.2% with vehicle, 5.0 ± 0.4% with A23187 (n = 3, P < 0.05), and 20.3 ± 0.1% with 5 mM Na3VO4/A23187 (n = 3, P < 0.05). GA treatment slightly, though not significantly, enhanced the A23187-induced release of AA, but did not affect the Na3VO4/A23187-induced release (Fig. 3A). The effect of 1 mM Na3VO4 on AA release was marginal with and without A23187, as reported previously (Taniguchi et al., 2007). Stimulation with 100 μM H2O2/A23187 for 1 hr released AA (approximately 15%), and GA treatment did not change the H2O2/A23187 response: 98.0% and 94.1% (% of control without GA, n = 2).

Next, we investigated the effect of GA treatment on AA release in HeLa cells. Stimulation of HeLa cells with 5 mM Na3VO4 did not markedly release AA with and without A23187 (data not shown). Since the releases of AA induced by PMA/A23187 and EGF/A23187 were mediated by cPLA2α’s activation in HeLa cells (Matsuzawa et al., 2009), we used these stimuli instead of Na3VO4. Treatment with 2 μM GA for 1 hr partially inhibited AA release induced by PMA/A23187, and completely inhibited the release by EGF/A23187 in HeLa cells (Fig. 3B). Sorafenib (BAY 43-9006, an inhibitor of Raf, Nexavar®)-sensitive Raf is involved in cPLA2α’s activation in HeLa cells (Matsuzawa et al., 2009). Treatment with 5 μM sorafenib for 30 min before the assay partially inhibited the PMA/A23187-induced release of AA: the value with sorafenib was 232 ± 25% (n = 4), which was significantly less (P < 0.05) than the control without sorafenib (381 ± 23%, n = 5). In HeLa cells treated with 2 μM GA for 18 hr, treatment with 5 μM sorafenib additionally inhibited the release: 141 ± 8% (n = 4), which was significantly lower than that with sorafenib alone. The release of AA without PMA/A23187 was 100% (n = 5) and 134 ± 23% (% of control, n = 5) without and with sorafenib, respectively. Like in PC12 cells, treatment with 2 μM GA by itself did not change cell morphology and cell detachment, and enhanced 50 μM H2O2-induced cytotoxicity at 24 hr after the treatment in HeLa cells (data not shown).

DISCUSSION

In this study, treatment with GA and 17-AAG at 2 μM, which alone did not cause cell detachment and LDH leakage, enhanced Na3VO4- and H2O2-induced cytotoxicity in PC12 cells. Treatment with the Hsp90 inhibitors at concentrations greater than 5 μM resulted in cytotoxicity, as shown previously in various neuronal cells including PC12 cells (Sano, 2001; Kim et al., 2003; Clark et al., 2009; Lu et al., 2009), and the inhibitors did not protect PC12 cells against the cytotoxicity at any concentrations tested from 1 nM. It has been reported that GA at less than 1 μM induced expression of the Hsp family including Hsp70 at 12 ~ 16 hr after treatment (Lu et al., 2002; Salehi et al., 2006) resulting in reduced cytotoxicity induced by oxidative stress (Sano, 2001; Romero et al., 2010) in PC12 cells. The different sensitivity to Hsp90 inhibitors may be explained by different characteristics of PC12 cells including levels of endogenous Hsp proteins depending on the subtype and passage of PC12 cells used (Romero et al., 2010).

It has been well established that stimuli increasing ceramide levels induce apoptosis and/or cell death, and inhibition of enzymes for ceramide metabolism cause and/or enhance cytotoxicity via accumulation of ceramide (Beljanski et al., 2010). 17-AAG caused cell death via ceramide signaling in colorectal cancer HT29 cells (Walker et al., 2010) and in human hepatic stellate cells (Myung et al., 2009). In contrast with ceramide, sphingosine-1-phosphate, a phosphorylated form of sphingosine, shows proliferative and anti-apoptotic effects (Posse de Chaves, 2006). In this study, we found that the formation of NBD-GlcCer was reduced by Hsp90 inhibitors with and without Na3VO4, and that treatment with Na3VO4 stimulated the formation of NBD-ceramipic acid, a counterpart of sphingosine, and the formation was markedly decreased by inhibitors of Hsp90 with and without Na3VO4, and that treatment with Na3VO4, stimulated the formation of NBD-ceramide to GlcCer, in addition to the possible accumulation of ceramide. The absolute amounts of NBD-GlcCer and NBD-ceramipic acid formed were approximately 15% and 6% of labeled NBD-ceramide, respectively, which may mask a possible increase of NBD-ceramide levels in Hsp90 inhibitor-treated cells. In addition to cell detachment by 1 mM Na3VO4, the response at 24 hr after 5 mM Na3VO4 treatment was...
enhanced by GA treatment. Thus, ceramide metabolism appeared to have an influence on Na3VO4-induced cytotoxicity, although the changes of ceramide metabolites in the cells treated with 1 mM Na3VO4 for 1 hr were limited in this study. Various cytotoxicity pathways have been proposed in cells whose Hsp90 is inhibited (Kim et al., 2003; Clark et al., 2009). The role of altered ceramide metabolism in these responses in Hsp90 inhibitor-treated cells remains to be elucidated.

AA metabolism couples with cytotoxicity in various cells including PC12 cells (Doroshenko and Doroshenko, 2007; Kurosawa et al., 2009; Nakamura et al., 2012). The inhibition of Hsp90 produces ROS, which can stimulate AA release via cPLA2α’s activation, as shown and HeLa cells (data not shown). We showed cytotoxicity including cell detachment in PC12 toxic 24 hr after stimulation, because A23187 alone ent of GA-sensitive factors, in PC12 cells. We could not of cPLA2α is regulated by a complex of Hsp90/p54 kinase et al in human mononuclear cells (Szántó et al., 2008; Clark et al., 2009). Ceramide metabolites such as C1P and sphingosine-1-phosphate are regulators for cPLA2α (Hirabayashi et al., 2004; Nakamura et al., 2006) and we showed changes in ceramide metabolism in Hsp90 inhibitor-treated PC12 cells. Thus, AA release may be involved in cytotoxicity induced by Hsp90 inhibitors. However, GA treatment changed neither the basal release nor the Na3VO4/A23187-induced release of AA in PC12 cells. The lack of an effect of GA on AA release in PC12 cells did not appear to be due to the conditions, since the treatment inhibited AA release in HeLa cells. The results suggest that 1) the cPLA2α-A2A pathway does not appear to be involved in GA-sensitive cytotoxicity, and 2) activation of cPLA2α with and without Na3VO4 was independent of GA-sensitive factors, in PC12 cells. We could not examine the role of AA released by A23187 in the cytotoxicity 24 hr after stimulation, because A23187 alone showed cytotoxicity including cell detachment in PC12 and HeLa cells (data not shown).

In HeLa cells, interestingly, treatment with GA inhibited the release of AA via cPLA2α’s activation, as shown in human mononuclear cells (Szántó et al., 2002). Tucker et al. (2008) reported that okadaic acid-induced activation of cPLA2α is regulated by a complex of Hsp90/p54 kinase that is sensitive to GA in mouse macrophages. These findings including ours suggest a role for Hsp90 in the regulation of cPLA2α activity in several cell types. There are at least three pathways for the activation of cPLA2α in HeLa cells: the Src-independent and sorafenib-insensitive pathway activated by EGF, and the Src-mediated and sorafenib-insensitive pathway and the sorafenib-sensitive pathway activated by PMA (Matsuazawa et al., 2009). In this study, GA treatment abolished the EGF-induced release of AA, and inhibited the PMA-induced release in the presence and absence of sorafenib, an inhibitor of the Raf family, in HeLa cells. GA treatment induces the degrada-

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