

Glucosidase II beta subunit (GluII β) plays a role in autophagy and apoptosis regulation in lung carcinoma cells in a p53-dependent manner

Worapong Khaodee¹ · Nichanan Inboot¹ · Suruk Udomsom^{2,3} · Warunee Kumsaiyai¹ · Ratchada Cressey^{1,4}

Accepted: 5 September 2017 / Published online: 19 September 2017
© International Society for Cellular Oncology 2017

Abstract

Purpose Glucosidase II plays a major role in regulating the post-translational modification of N-linked glycoproteins. Previously, we found that the beta subunit of glucosidase II (GluII β) levels are significantly increased in lung carcinoma tissues, indicating a potential role in lung tumorigenesis. Here, we investigated the role of GluII β in the regulation of autophagy and apoptosis in lung carcinoma- and immortalized human bronchial epithelial-derived cells.

Methods A selective glucosidase II inhibitor, bromoconduritol, was used to inhibit GluII enzyme activity and a siRNA-based technology was used to suppress the expression of the GluII β encoding gene *PRKCSH* in lung carcinoma cells differing in p53 status. Cell viability was assessed using a MTT assay, cell cycle progression was assessed using flow cytometry, autophagy was assessed using Western blotting and apoptosis was assessed using an annexin V-FITC/PI double labeling method. **Results** We found that GluII β inhibition resulted in the induction of autophagy in all cell lines tested, but apoptosis in only wild-type p53 cells. We also found that GluII β inhibition dose-dependently decreased activation of the EGFR/RTK

and PI3K/AKT signaling pathways. Although the apoptosis inducing effect of GluII β inhibition appeared to be p53-dependent, we found that a combined treatment with lysosomal inhibitors to block autophagy enhanced the apoptotic effect of GluII β inhibition in both wild-type p53 and p53-null cells. **Conclusions** Our data indicate that GluII β inhibition results in autophagy and apoptosis in lung carcinoma-derived cells, supporting the hypothesis that this enzyme may play a role in blocking these two tumor suppressive processes. Since blocking autophagy by lysosomal inhibitors enhanced the apoptosis-inducing effect of bromoconduritol, independent of p53 status, their combined use may hold promise for the treatment of cancer, particularly lung cancer.

Keywords Apoptosis · Autophagy · Glucosidase II beta subunit (GluII β) · *PRKCSH* gene · p53

1 Introduction

Previously, we detected high concentrations of the endoplasmic reticulum (ER)-resident protein glucosidase II beta subunit (GluII β) in a high proportion of lung carcinoma tissues, indicating a role in tumorigenesis [1]. This protein and/or its encoding gene are also known as *PRKCSH*, 80K-H, hepatocystin, PCLD, PLD1, G19P1 or AGE-R2. GluII β is a beta subunit of glucosidase II (GluII), which is involved in the quality control of N-linked glycoprotein folding. The heterodimeric GluII complex, consisting of a catalytic alpha subunit and a regulatory beta subunit, is responsible for the removal of the two innermost glucose residues from N-linked oligosaccharides on newly synthesized glycoproteins. Protein folding is an error-prone process and, therefore, a quality control process is in place in the ER to evaluate the structural integrity of maturing proteins. Properly folded proteins are permitted to

✉ Ratchada Cressey
Ratchada.cr@cmu.ac.th

¹ Division of Clinical Chemistry, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand

² Biomedical Engineering Program, Faculty of Engineering, Chiang Mai University, Chiang Mai, Thailand

³ Biomedical Engineering Center, Chiang Mai University, Chiang Mai, Thailand

⁴ MT Cancer Research Unit, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand

leave the ER and to traffic to various locations within the cell to perform their activities, whereas misfolded proteins are targeted for retention and subsequent degradation. GluII is responsible for the release of N-linked glycoproteins from the ER, whereas the quality control gatekeeper UDP-glucose:glycoprotein glucosyltransferase 1 (UGT1) promotes their retention [2]. A balance between GluII and UGT1 activity is thus crucial for a proper quality control of proteins in the ER.

Mutations in the GluII β encoding gene have been associated with autosomal dominant polycystic liver disease (ADPLD) [3] and alternatively spliced transcripts encoding distinct isoforms have been reported [4]. Although a role of GluII β in oncogenesis has not been reported before, a number of studies have reported the occurrence of gene mutations [5] and increased protein expression levels in human cancers [6], as well as alterations in cellular localization and phosphorylation status in response to growth factor stimulation [6, 7]. Suppression of GluII β expression has been reported to cause autophagy through the mammalian target of rapamycin (mTOR) pathway [8] and a recent study has shown that GluII β may also be involved in the initiation of apoptosis in plant cells [9].

Both apoptosis and autophagy play important roles in cancer. Apoptosis is established as the major mechanism of programmed cell death and is estimated to eliminate approximately 60 billion cells per day in order to maintain normal tissue homeostasis [10]. Deregulation of apoptosis leads to the accumulation of abnormal cells and, ultimately, cancer development. Autophagy is a process aimed at degrading damaged intracellular macromolecules in order to provide cells with energy under nutrient-starved conditions. Autophagy has been reported to play both positive and negative roles in cancer development. On one hand, autophagy suppresses tumor formation by removing damaged organelles/proteins and limiting cell growth and genomic instability. When disrupted, it can promote and accelerate tumorigenesis [11–13]. Autophagy may be inhibited by multiple oncogenic products, such as PI3K, AKT, BCL-2 and mutant p53, and, therefore, inhibition of autophagy may be considered as a potential oncogenic event [14]. On the other hand, due to inherent deficiencies in their microenvironment and the increased metabolic and biosynthetic demands imposed by deregulated proliferation, cancer cells rely on autophagy for their survival more than normal cells [15].

Lung cancer is the leading cause of cancer-related deaths worldwide. Non-small cell lung cancer (NSCLC) comprises 80–85% of all lung cancers, [16] and most patients present with metastasis [17, 18]. A major barrier to curative therapy in lung cancer is the deregulation of cell death signaling processes [19, 20]. Of these processes, apoptosis has so far been the most extensively studied. Multiple studies have shown that deficits in the apoptotic machinery can lead, not only to

abnormal proliferation, but also to insensitivity to cytotoxic drugs [21]. Exploitation of autophagy may lead to improved therapeutic options beyond current therapies. It is generally accepted now that the tumor suppressor protein p53 plays a major role in the radio-sensitivity of lung cancer cells [22]. Here, we aimed to investigate the role of GluII β in autophagic and apoptotic cell death of lung carcinoma-derived cells differing in p53 status.

2 Material and methods

2.1 Chemicals

The selective glucosidase II inhibitor bromoconduritol [23] and antibodies directed against GluII β , human p53 and LC3 were obtained from Santa Cruz Biotechnology, Inc. (Texas, USA). Horseradish peroxidase-conjugated anti-rabbit, goat or mouse immunoglobulins (IgGs) were purchased from DakoCytomation (Denmark). Clarity™ ECL Western blotting substrate was obtained from Bio-Rad Laboratories (California, USA). Peptastatin A and E64d were purchased from AMRESCO Life Science (Ohio, USA).

2.2 Cell lines and culture conditions

A549, BEAS-2B and H1299 cells were obtained from the American Tissue Culture Collection (ATCC). A549 human lung carcinoma cells were maintained in DMEM medium, whereas p53-deficient H1299 human lung carcinoma cells were maintained in RPMI-1640 medium. Both DMEM and RPMI-1640 were supplemented with 10% fetal bovine serum (FBS) (v/v), 100 units/ml penicillin and 100 μ g/ml streptomycin (Gibco-Thermo Fisher Scientific, Massachusetts, USA). Primary immortalized human bronchial epithelial BEAS-2B cells were maintained in LCH-9 medium (Gibco-Thermo Fisher Scientific, Massachusetts, USA).

2.3 Cell viability assay

Cells (approximately 1×10^4 cells/well) seeded in 96-well plates were grown overnight and subsequently treated with various concentrations of bromoconduritol (0–500 μ g/ml) in 200 μ l medium per well at 37 °C and 5% CO₂. After 72 h, 20 μ l 3–4,5 dimethyl thiazol 2,5 diphenyl tetrazolium bromide (MTT) solution (5 mg/ml) was added to each well and incubated for 4 h. The resulting violet formazan precipitate was solubilized by the addition of 100 μ l dimethyl sulfoxide (DMSO) after which the absorption was measured at 595 nm (Emax Plus microplate reader, Molecular Devices, California, USA).

2.4 RNAi treatment

siRNA transfections were conducted accordingly to the Santa Cruz siRNA transfection protocol. Approximately 2×10^5 cells/well were seeded and cultured in a 6-well tissue culture plate overnight. Next, 100 μ l siRNA duplex solution (sc-29598, Santa Cruz Biotechnology, Texas, USA) was mixed with 100 μ l transfection reagent (sc-29528, Santa Cruz Biotechnology, Texas, USA) and incubated for 45 min at room temperature. The resulting mixture was mixed gently with 800 μ l incomplete medium (DMEM for A549 and RPMI-1640 for H1299), added to the cells and incubated for 5 h at 37 $^{\circ}$ C in a CO₂ incubator. Next, 1 ml normal growth medium containing 20% FBS (v/v) and antibiotics (200 units/ml penicillin and 200 μ g/ml streptomycin) was added to the transfected cells without removing the transfection mixture and incubated for an additional 18–24 h. Next, the culture medium was replaced with fresh medium (DMEM for A549 and RPMI-1640 for H1299) containing 10% FBS (v/v), 100 units/ml penicillin and 100 μ g/ml streptomycin, after which the transfected cells were cultured for another 24 h before subjecting to further tests.

2.5 Western blot analysis

Treated cells were lysed in sodium dodecyl sulfate (SDS) lysis buffer [0.5 M Tris-HCl pH 6.8, 2% SDS (w/v) and 10% glycerol (v/v)] and heated at 95 $^{\circ}$ C for 10 min. The resulting cell lysates were centrifuged at 10,000 \times g for 15 min at 4 $^{\circ}$ C, after which the supernatants were collected and their protein content was determined using a BCA protein assay kit (Pierce Biotechnology, Illinois, USA). Protein lysates (25 μ g) from each treatment condition were resolved in SDS polyacrylamide gels under reducing conditions and electro-transferred to PVDF membranes (Pall Corporation, New York, USA). After blocking with 5% non-fat milk in Tris buffered saline (TBS) containing 0.05% Tween-20 (TBS-T), the membranes were incubated with primary antibodies for 1 h at room temperature. The bound antibodies were detected using horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat or rabbit IgGs for 1 h. After extensive washing, immunoreactive proteins were visualized using a chemiluminescence-based procedure in conjunction with a Clarity ECL detection kit (Biorad Laboratories, California, USA) and X-ray films (Kodak, New York, USA). The detected bands were quantified using ImageJ software [24]. Next, the membranes were stained with 0.1% Coomassie Blue R250 in 10% acetic acid, 40% methanol and 50% H₂O for 1 h in order to assess the total amount of protein loaded into each lane. The membranes were de-stained in tap water (7 times, 10 min each) until the background was clear.

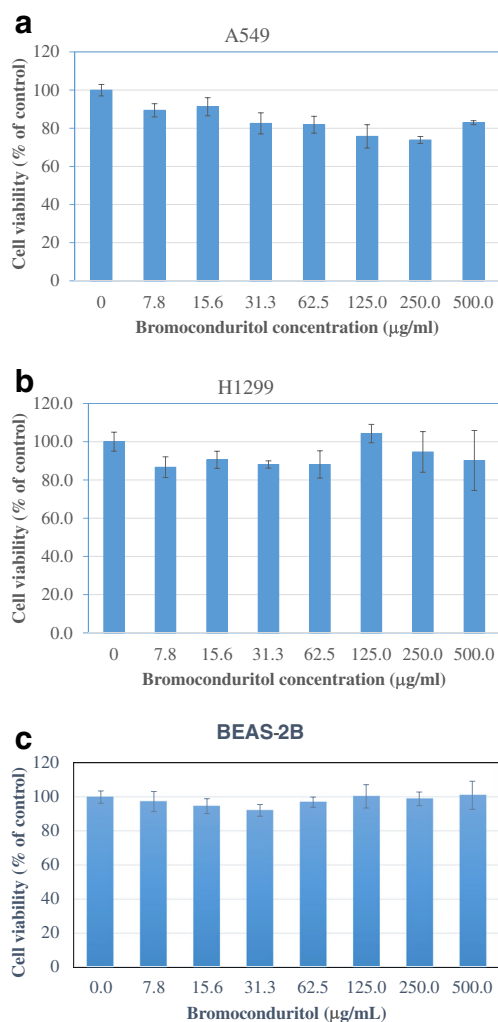


Fig. 1 Viability of A549 (a), H1299 (b) and BEAS-2B (c) cells after treatment with various concentrations of bromoconduritol for 72 h

2.6 Cell cycle analysis

Cells were cultured with various concentrations of bromoconduritol (0, 1, 5, 10 μ g/ml) for 24 h and, subsequently, subjected to cell cycle analysis using a Muse™ flow cytometer, according to the Muse™ Cell Cycle Kit users guide (Merck-Millipore, Massachusetts, USA). The Muse™ Cell Cycle Assay Kit encompasses a premixed reagent which includes propidium iodide (PI) to discriminate cells at different stages of the cell cycle, based on differential DNA content in the presence of RNase to increase the specificity of DNA staining.

2.7 Apoptosis assay

Approximately 4×10^4 cells/well were seeded in a 96-well plate and grown overnight to reach 70–80% confluence and, subsequently, treated with different concentrations of bromoconduritol for 24 h. Treated cells from triplicated wells

Table 1 Cell cycle distribution after treatment with bromoconduritol for 24 h

Cells	Bromoconduritol concentration ($\mu\text{g/ml}$)	Cell cycle distribution (%)		
		G0/G1	S	G2/M
A549	0	59.3 \pm 2.26	19.6 \pm 5.51	20.9 \pm 2.20
	1	53.5 \pm 1.21	20.3 \pm 3.46	26.1 \pm 4.21
	5	54.1 \pm 3.29	24.9 \pm 2.27	20.8 \pm 1.21
	10	49.8 \pm 6.22	29.2 \pm 2.86	21.0 \pm 4.26
H1299	0	55.4 \pm 3.23	22.1 \pm 2.46	22.4 \pm 1.29
	1	60.6 \pm 8.21	19.2 \pm 5.23	20.1 \pm 3.21
	5	55.3 \pm 4.16	21.3 \pm 2.96	23.4 \pm 4.21
	10	61.1 \pm 9.27	18.1 \pm 6.23	20.4 \pm 3.29

were pooled and analyzed using a Muse™ Cell Analyzer (Merck-Millipore, Massachusetts, USA) according to the

manufacturer's instructions using Muse™ Annexin-V and Dead Cell reagent, respectively (Merck-Millipore,

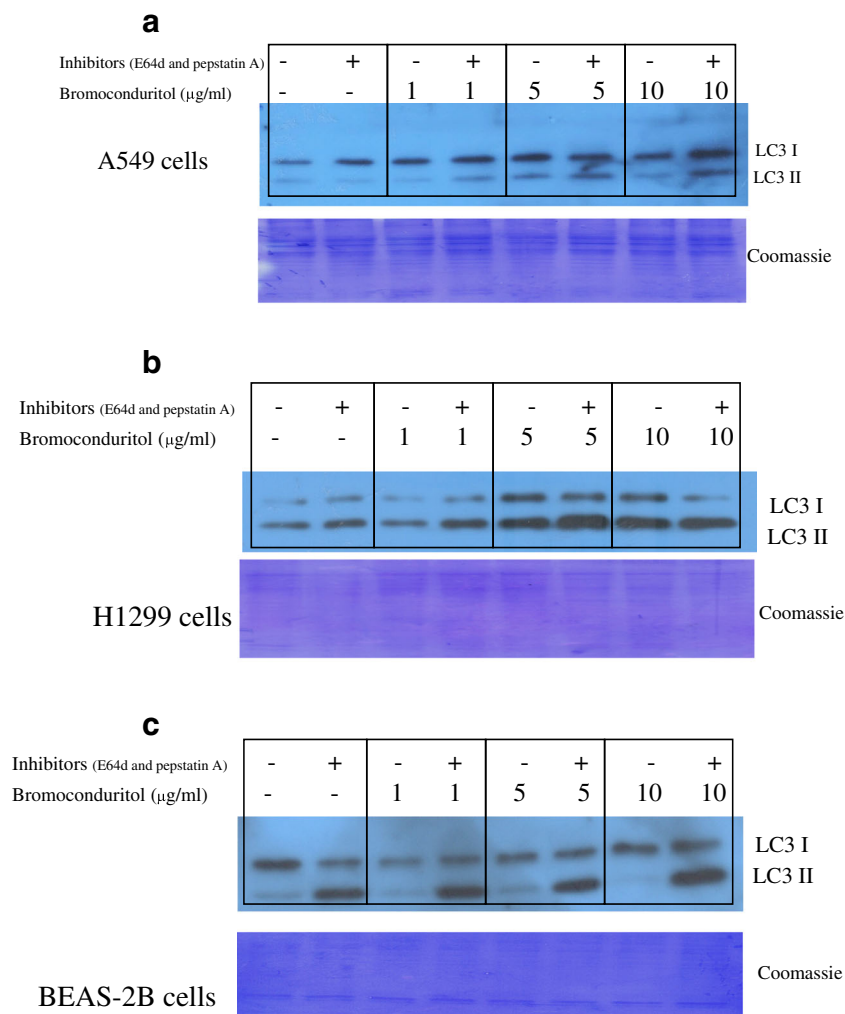


Fig. 2 Autophagy induction after treatment with bromoconduritol in A549 (a), H1299 (b) and BEAS-2B (c) cells and after siRNA-mediated suppression of *PRKCSH* gene expression in A549 (d) and H1299 (f) cells. Bar graphs represent intensities of LC3-II bands relative to control cells quantified by ImageJ from A549 (e) and H1299 (g) transfected cells. Cells were treated with bromoconduritol at concentration of 0, 1, 5 and 10 $\mu\text{g/ml}$ for 24 h or transfected with siPRKCSH for 48 h in the presence

or absence of the lysosomal inhibitors E-64d (10 $\mu\text{g/ml}$) and pepstatin A (10 $\mu\text{g/ml}$), which were added to the culture media 4 h before cell harvesting. Western blots were probed with anti-LC3-I and anti-LC3-II antibodies after which the PVDF membranes were subjected to Coomassie blue staining to verify the amount of protein loaded into each lane as internal control

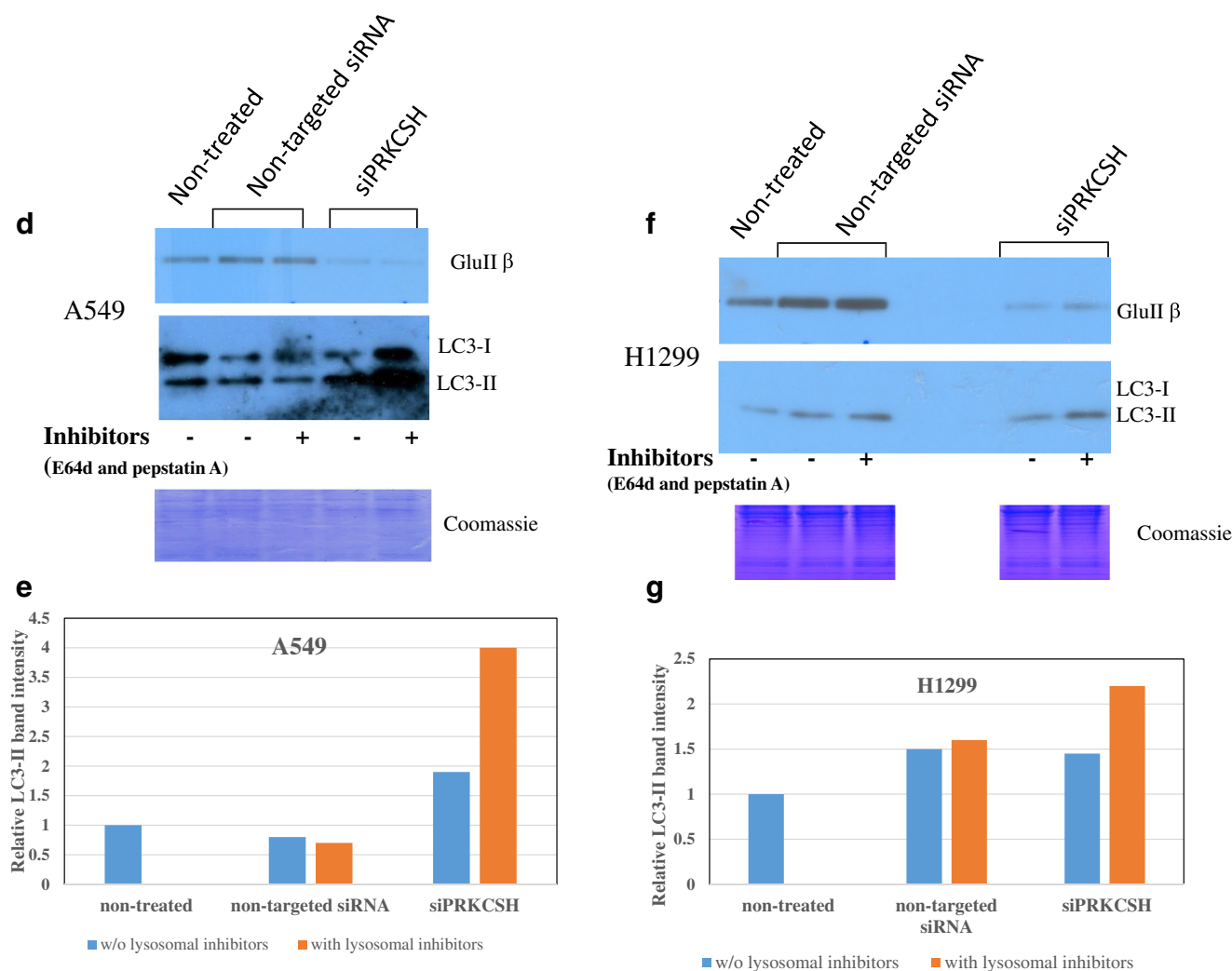


Fig. 2 (continued)

Massachusetts, USA). Cells treated with 5 μM Apoptosis Activator 2 (sc-202956, Santa Cruz Biotechnology, Texas, USA) were used as a positive control for apoptosis induction.

2.8 Cell signaling pathway analysis

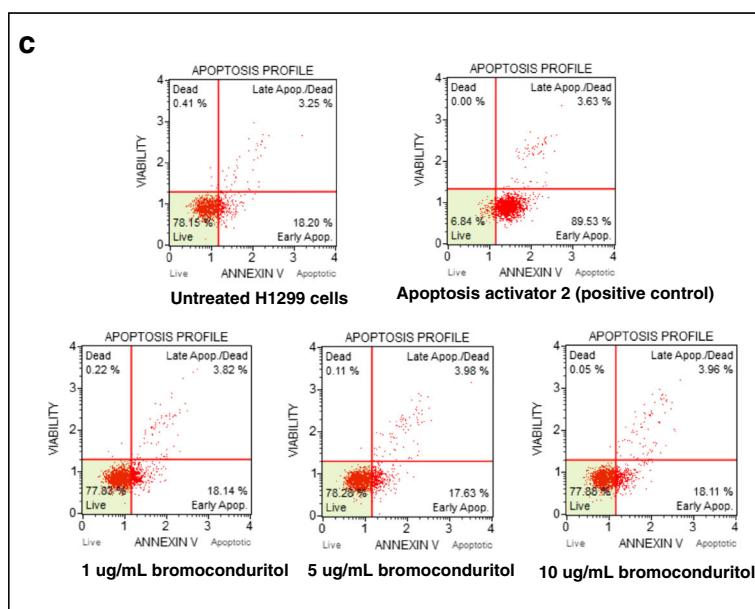
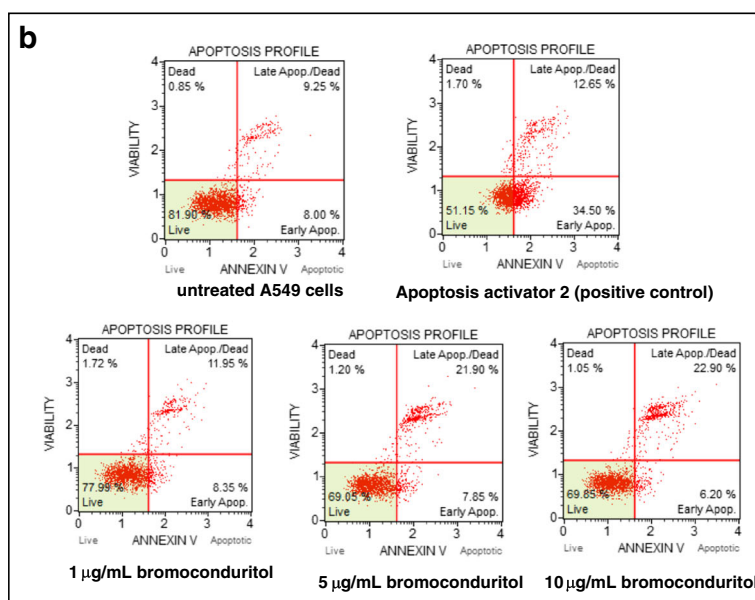
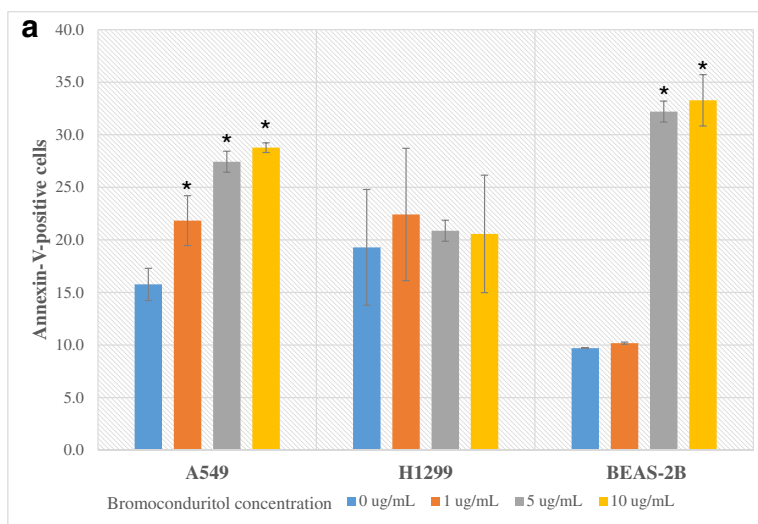
After 24 h of treatment with various concentrations of bromoconduritol (0, 1, 5, 10 μg/ml), approximately 7.5×10^4 treated and untreated cells were centrifuged and re-suspended in a mixture of Assay Buffer and Fixation Buffer (1:1), and incubated for 5 min on ice. Next, the cells were centrifuged and re-suspended in Assay Buffer and subjected to incubation with antibodies for 30 min in the dark at room temperature. The Muse EGFR-RTK Activation Dual Detection Kit (Merck Millipore, Massachusetts, USA) used includes two directly conjugated antibodies, a phospho-specific anti-phospho-EGFR (Tyr1173)-Alexa Fluor®555 and an anti-EGFR-PECy5 conjugated antibody to measure phosphorylated and total levels of EGFR expression,

respectively. The Muse PI3K Activation Dual Detection Kit (Merck Millipore) includes a phospho-specific anti-phospho-Akt (Ser473)-Alexa Fluor®555 and an anti-Akt, PEcy5 conjugated antibody to measure phosphorylated and total levels of AKT, respectively. These two-color kits are designed to measure the extent of EGFR phosphorylation relative to the total EGFR expression and of AKT phosphorylation relative to the total AKT expression in any given cell population. After antibody staining, the cells were suspended in Assay Buffer and transferred to the Muse Cell Analyzer (Merck Millipore, Massachusetts, USA).

2.9 Statistical analysis

Statistical analyses were performed using SPSS software version 15 (SPSS, Inc., Chicago, IL, USA). Comparison of different groups was performed using the Mann-Whitney U test, with $p < 0.05$ considered to be significant.

Fig. 3 Apoptosis induction after treatment with bromoconduritol for 24 h. **a** Mean and standard deviation (SD) of total apoptosis as percent from three independent experiments each. A *p* value of < 0.05 (*) was considered significant relative to the non-treated cells using the Mann Whitney U test. **b–d** Representative flow cytometry plots showing annexin V and propidium iodide (PI) staining in A549, H1299 and BEAS-2B cells, respectively



3 Results

3.1 GluII β inhibition does not affect cell viability or cell cycle distribution

In order to test whether inhibition of GluII β by bromoconduritol would exert any effect on cell viability or cell cycle distribution, normal human bronchial epithelial-derived BEAS-2B cells and the human lung carcinoma-derived cell lines A549 and H1299 with different p53 statuses were treated with various concentrations of bromoconduritol for 72 h. By doing so, we found that treatment with bromoconduritol at concentrations between 7.8 and 500 $\mu\text{g/ml}$ did not significantly affect the cell viability (Fig. 1a–c) and/or cell cycle distribution of any of the cell types tested (Table 1).

3.2 GluII enzyme suppression and *GluII β* gene silencing induce autophagy

To test the contribution of GluII β to autophagy, cells were treated with various concentrations of bromoconduritol (1, 5 and 10 $\mu\text{g/ml}$) in the presence or absence of E64d and pepstatin A. E64d and pepstatin A are two autophagy inhibitors that act by suppressing lysosomal proteases. During autophagy, soluble LC3-I is converted to lipid bound LC3-II, which is associated with the formation of autophagosomes. Autophagosomes fuse with lysosomes to form autolysosomes, after which intra-autophagosomal components are degraded by lysosomal enzymes. Thus, lysosomal turnover of the autophagosomal marker LC3-II reflects autophagic activity [25]. By comparing levels of LC3-II between treatments in the presence and absence of E64d and pepstatin A, the magnitude of autophagic flux can be estimated [26]. Using Western blotting, we found that the accumulation of LC3-II in all cell lines tested (Fig. 2a–c) was significantly increased in the presence of E64d and pepstatin A following bromoconduritol treatment compared to control cells, thus indicating their autophagic induction. Although, the viability of the siPRKCSH transfected cells was not significantly different from that of the vehicle control transfected cells (as observed microscopically, data not shown), we conclude that knockdown of the *PRKCSH* gene, which encodes beta subunit of the enzyme (GluII β), caused a conversion from LC3-I to LC3-II. The bar graphs in Fig. 2 represent intensities of LC3-II bands from A549 (2e) and H1299 (2g) transfected cells relative to control cells quantified by ImageJ. The results indicate that blocking of lysosomal enzymes by E64d and pepstatin A causes a substantial accumulation of LC3-II in both A549 (Fig. 2d, e) and H1299 (Fig. 2f, g) cells, which is an indication of autophagy induction. Taken together, we found that either suppression of GluII enzyme activity by bromoconduritol or suppression of *GluII β* gene expression by siRNA can induce autophagy in lung carcinoma-derived cells.

3.3 GluII β suppression by bromoconduritol induces apoptosis in p53 wild-type cells, but not in p53-null cells

In order to investigate the effect of GluII β suppression on apoptosis, cells treated with various concentrations of bromoconduritol were assessed for apoptotic cell death using a Muse™ Annexin V & Dead Cell Kit. We found that suppression of GluII β activity by bromoconduritol caused an increase in apoptotic cell death in A549 and BEAS-2B cells, both carrying wild-type p53, in a dose-dependent manner (Fig. 3a). Conversely, no significant change in apoptotic cell death was observed in p53-null H1299 cells. Representative flow cytometry plots based on annexin V and propidium iodide (PI) staining of A549, H1299 and BEAS-2B cells treated with various concentrations of bromoconduritol are shown in Fig. 3b–d, respectively. Cells treated with 5 μM of Apoptosis Activator 2, which is a cell-permeable indole compound that activates caspases, were used as positive controls for apoptosis induction. From the results obtained we conclude that suppression of GluII β results in apoptosis in wild-type p53 cells (A549, BEAS-2B), but not in p53-null cells (H1299).

3.4 GluII β suppression causes decreased activation of the PI3K and EGFR pathways

Since the epidermal growth factor receptor (EGFR) has frequently been found to be deregulated in lung carcinomas [27, 28] and since the PI3K/AKT pathway is one of the most important EGFR downstream signaling pathways, the ability of bromoconduritol to modulate the EGFR-PI3K/AKT pathway was investigated. Cells treated for 24 h with various concentration of bromoconduritol were stained with both anti-phospho-EGFR (Tyr1173) and anti-EGFR antibodies, or anti-phospho-Akt (Ser473) and anti-Akt antibodies in a multiplex reaction. Stained cells were analyzed using a Muse Cell Analyzer and through statistical value calculations, cells were classified as relative percentage of each population (inactive, active and not-expressing), compared to the total cell population. We found that treatment with bromoconduritol caused a significant decrease in activation of the EGFR and PI3K/AKT pathways (Figs. 4a and 5a). Representative flow cytometry plots showing EGFR phosphorylation and AKT phosphorylation in A549 and H1299 cells treated with various concentrations of bromoconduritol are depicted in Figs. 4b–c and 5b–c, respectively.

3.5 Autophagic flux inhibition enhances apoptosis induction by bromoconduritol

To test whether blocking of the autophagic flux may have any effect on the apoptosis inducing efficacy of bromoconduritol, cells were treated with various concentrations of bromoconduritol in the presence or absence of the lysosomal inhibitors pepstatin A

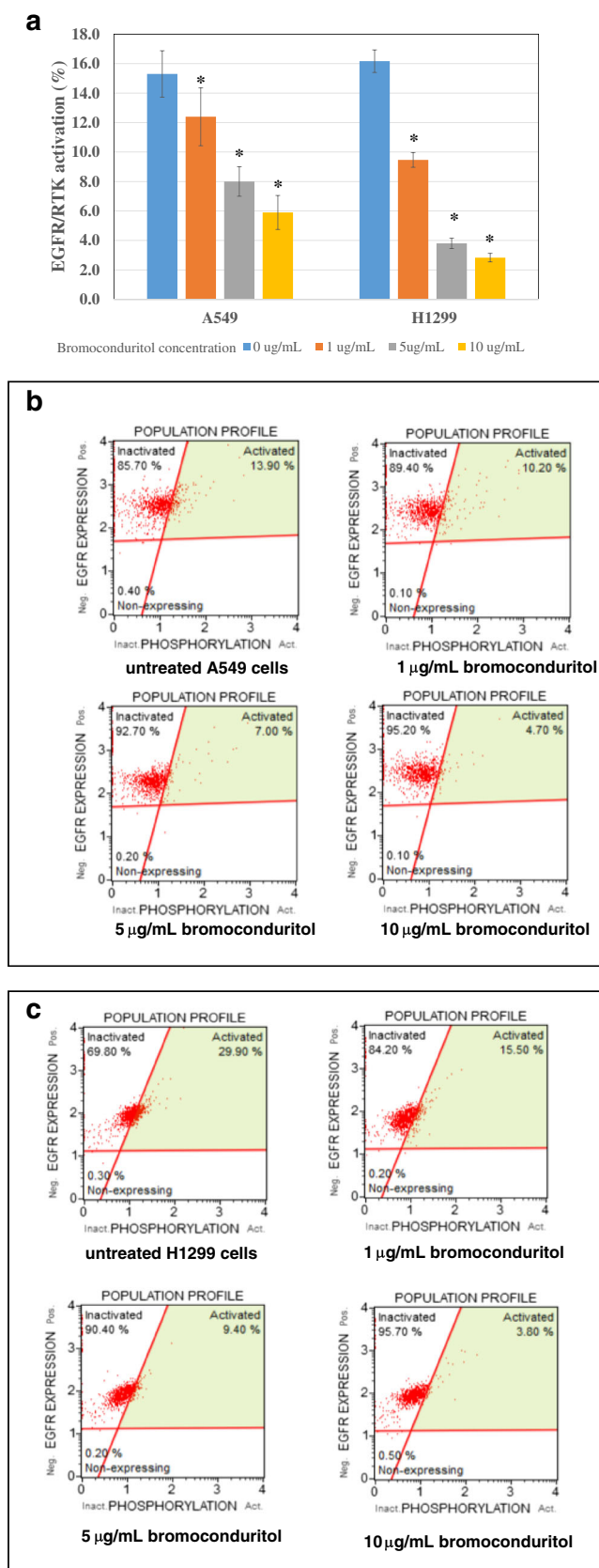


Fig. 4 Significant decrease in activation of the EGFR/RTK signalling pathway after treatment with bromoconduritol for 24 h. **a** Mean and standard deviation (SD) of EGFR phosphorylation from three independent experiments. A p value of < 0.05 (*) was considered significant relative to the non-treated cells using the Mann Whitney U test. **b-c** Representative flow cytometry plots showing EGFR expression and phosphorylation in A549 and H1299 cells, respectively

and E64d (10 µg/ml each). By doing so, we found that a combined treatment with lysosomal inhibitors efficiently enhanced the apoptosis inducing effect of bromoconduritol in both A549 (Fig. 6a) and H1299 (Fig. 6b) cells. Representative flow cytometry plots showing annexin V and PI staining of A549 and H1299 cells treated with bromoconduritol and lysosomal inhibitors are depicted in Fig. 6c–d, respectively.

4 Discussion

We found that treatment of the wild-type p53 cells and p53-null cells with the selective GluII inhibitor bromoconduritol dose-dependently increased the turnover rate of LC3-II when lysosomal enzymes were inhibited by E64d and pepstatin A. siRNA-mediated silencing of the *PRKCSH* gene, which encodes GluII β , induced a similar response. Since glucosidase II comprises 2 subunits, i.e., the alpha catalytic subunit (GluII α) and the beta regulatory subunit (GluII β), our results suggest that suppression of GluII β activity/expression alone is sufficient to inhibit its total enzyme activity. Our results indicate that inhibition of glucosidase II activity, either by selective GluII inhibition or by suppression of *PRKCSH* gene expression, resulted in initiation of autophagy in both wild-type p53 cells and p53-null cells. However, suppression of GluII β only triggered apoptosis in wild-type p53 cells. Since only one p53-null cell line was used in this study, it remains to be established whether other types of p53 defective cells will show a similar response. Autophagy induction in response to GluII β inhibition has previously been reported [8], but to our knowledge this is the first report showing apoptosis induction in response to GluII β inhibition in lung carcinoma-derived cell lines.

The observation that suppression of GluII β resulted in the induction of autophagy and apoptosis led us to hypothesize that GluII β may play a role in suppression of these two critical tumor-associated processes. It may be envisioned that activation of GluII β expression helps tumor cells to escape from autophagy and apoptosis and, thus, to advance their progression. Indeed, this may be the reason why in previous reports an increased level of GluII β was observed in various tumor tissues [1, 6]. The beta regulatory subunit of glucosidase II, GluII β , is involved in the quality control of the folding and maturation of N-linked glycoproteins. This group of proteins encompasses receptor tyrosine kinases (RTK), such as ErbB2 and EGFR [29], which can initiate a number of downstream

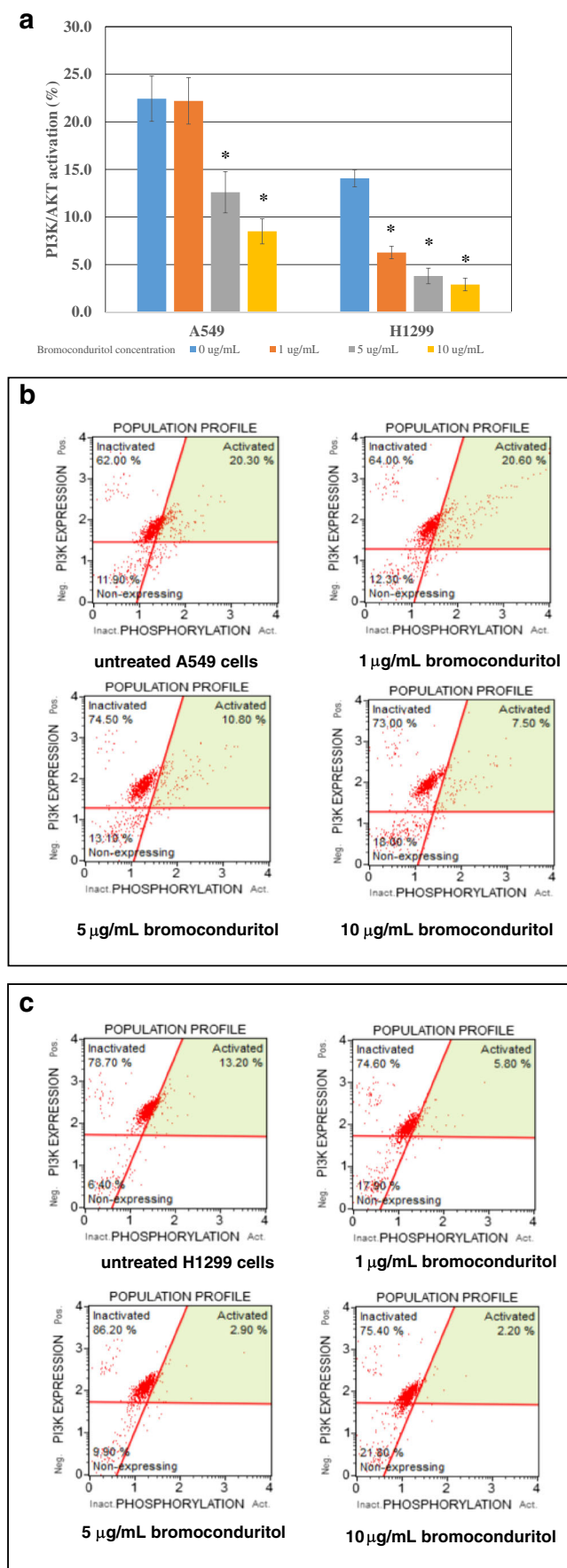


Fig. 5 Significant decrease in activation of the PI3K/AKT signalling pathway after treatment with bromoconduritol for 24 h. **a** Mean and standard deviation (SD) of AKT phosphorylation from three independent experiments. A *p* value of < 0.05 (*) was considered significant relative to the non-treated cells using the Mann Whitney U test. **b–c** Representative flow cytometry plots showing AKT expression and phosphorylation in A549 and H1299 cells, respectively

signaling cascades resulting in both anti-apoptotic [30] and anti-autophagic [31] cellular programs. Inhibition of N-linked glycosylation has been shown to prevent both ligand-induced activation of EGFR as well as constitutive EGFR-VIII signaling [32]. More recently, it has been shown that EGFR activity may be regulated by specific glycosylation side chain modifications [33, 34] and may require glycosylation as part of the transitional state conformation required for signaling [35]. Therefore, we hypothesize that tumor cells with an abnormal high level of GluII β activity may aberrantly

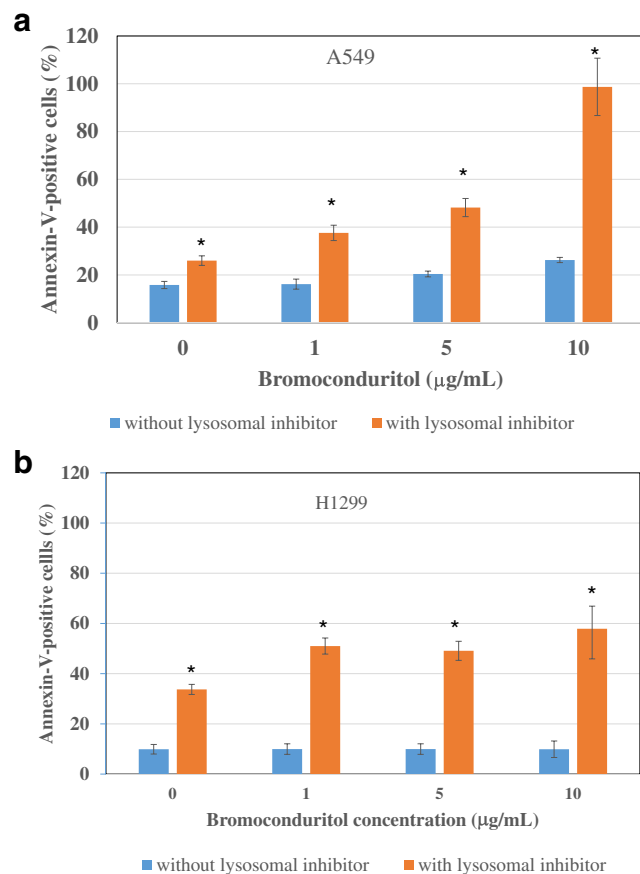


Fig. 6 Enhancement of bromoconduritol-induced apoptosis in the presence of lysosomal inhibitors (pepstatin A and E64d). **a–b** Mean and standard deviation (SD) of total apoptosis in percent from three independent experiments in A549 and H1299 cells, respectively. A *p* value of < 0.05 (*) was considered significant relative to the non-treated cells using the Mann Whitney U test. **c–d** Representative flow cytometry plots showing annexin V and propidium iodide (PI) staining in A549 and H1299 cells, respectively. Cells were treated with bromoconduritol at concentrations of 0, 1, 5 and 10 $\mu\text{g/ml}$ for 24 h in the presence or absence of the lysosomal inhibitors E-64d and pepstatin A (10 $\mu\text{g/ml}$ each)

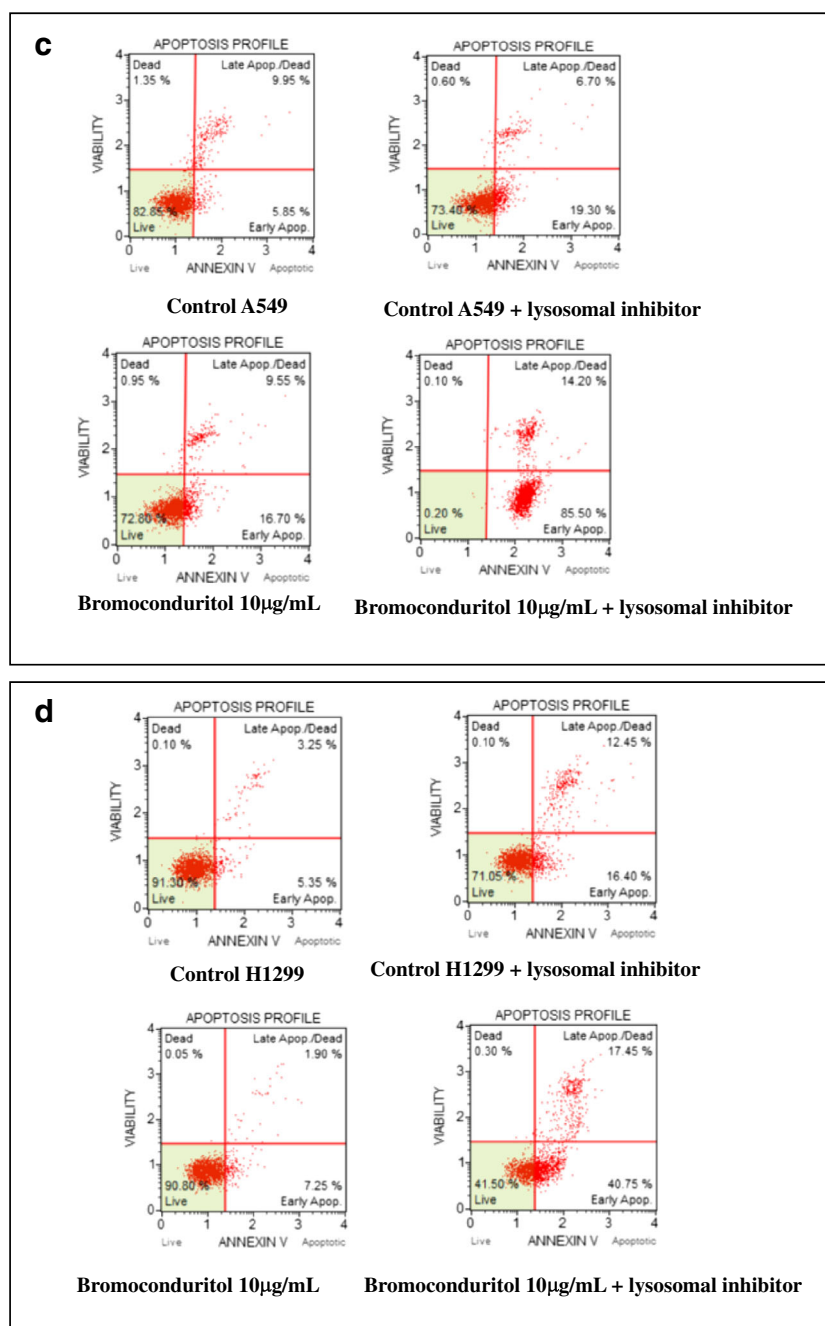


Fig. 6 (continued)

promote N-linked glycosylation and, thus, overactivation of the EGFR signaling pathway and, consequently, apoptosis and autophagy inhibition. In agreement with this hypothesis, we found that GluII β activity suppression caused a significant reduction in EGFR and its downstream PI3K/AKT pathway activation in both A549 and H1299 cells in a dose-dependent manner. These results substantiate the notion that GluII β overexpression in tumor cells may promote tumor growth by suppressing apoptosis and autophagy through activation of the EGFR/RTK and PI3K/AKT pathways. Correspondingly, it has been demonstrated that silencing of the *PRKCSH* gene

enhanced the apoptosis inducing effect of gefitinib, a tyrosine kinase inhibitor, in a gefitinib-resistant NSCLC cell line [36].

Bromoconduritol (6-bromo-3,4,5-trihydroxycyclohex-1-ene) is an active site-directed irreversible inhibitor of glucosidases II with an IC₅₀ of 41 μ M (approximately 8.6 μ g/ml) [23]. An analysis of L₆ myoblasts has shown that bromoconduritol at a concentration of 50 μ g/ml can significantly inhibit myoblast fusion, a process that is required for the formation of skeletal muscles, whereas no inhibition of cell growth was observed up to a concentration of 250 μ g/ml [37]. Also, it has been reported that 200 μ g/ml

bromoconduritol is able to delay intracellular transport of secretory glycoproteins in human hepatoma cell cultures [38]. Interestingly, it has been found that treatment of murine sarcoma L-1 cells with bromoconduritol at a concentration below its IC₅₀ (0.5 and 2 μ g/ml) for 20–24 h significantly inhibits their ability to colonize in the lung of Balb/c-mice after intravenous injection [39]. We found that bromoconduritol at a concentration of 1–10 μ g/ml was able to induce autophagy and apoptosis. However, no significant impact on cell viability was seen when assessed by MTT assay at a concentration of up to 500 μ g/ml. As a double-edged sword, autophagy plays a dual role in many diseases including cancer. It has been found that autophagy can act both as a tumor suppressor and as a promoter of cancer cell survival [40]. We hypothesized that autophagy may help the survival of cells in response to bromoconduritol treatment, and tested this hypothesis by investigating the impact of autophagy inhibition on cell survival and apoptosis. We found that blocking the autophagic flux by the lysosomal inhibitors E64d and pepstatin A reduced cell survival by enhancing the apoptosis-inducing effect of bromoconduritol.

During autophagy, autophagosomes fuse with lysosomes after which the content is degraded and recycled. Lysosomes contain various types of enzymes such as peptidases, phosphatases and proteases. Among these enzymes, cathepsins constitute a major class of lysosomal proteases which are cleaved from pro-cathepsins and are activated in the lysosomes [41]. E64d and pepstatin A are two autophagy inhibitors that function by suppressing lysosomal proteases. E64d is a membrane-permeable inhibitor of cathepsins B, H and L, whereas pepstatin A is an inhibitor of cathepsins D and E [42]. The impairment of autophagy by inhibition of cathepsins B and L has been reported to induce cell death through lysosomal dysfunction [26]. Our observation that the blocking of autophagy by E64d and pepstatin A enhances the apoptosis-inducing effect of bromoconduritol by up to 100% (at concentrations that otherwise showed no effect on cell viability) in both wild-type p53 cells and p53-null cells indicates that these compounds may be used in cancer treatment. It still remains to be determined, however, whether bromoconduritol combined with lysosomal enzyme inhibitors has any toxic side-effects on normal cells.

p53 is the most commonly inactivated gene in human cancers, although some cancers remain wild-type p53 [43]. It functions as a signal transduction integrator that is activated by various stressors as diverse as DNA damage, ischaemia-reperfusion and nutritional stress. Both the extrinsic and intrinsic apoptotic pathways have been shown to be activated by p53. In contrast to apoptosis upregulation by p53, cytoplasmic and nuclear p53 play contradictory roles in regulating autophagy. Cytoplasmic p53 inhibits autophagy through activation of mTOR

signaling via the inactivation of AMP kinase [44], while nuclear p53 activates autophagy by transcriptional activation of DRAM (damage-regulated autophagy modulator), which promotes the formation of autophago-lysosomes [45]. The complicated role of p53 in regulating autophagy and apoptosis turns it into an important but complex target for cancer therapy [46]. Our present work indicates that inhibition of GluII β activity results in apoptosis induction in only wild-type p53 cells, and in autophagy in both wild-type p53 cells and p53-null cells. Although mTOR inhibition has been reported to constitute a definitive step in the induction of autophagy by GluII β deficiency [8], its mediation does not seem to be p53 dependent.

In conclusion, we found that inhibition of GluII β activity results in the initiation of autophagy and apoptosis, which in part, may be brought about through reduction of the EGFR/RTK and PI3K/AKT signaling pathways. This observation suggests that GluII β may facilitate tumor growth through a positive regulation of these two signaling pathways during tumor development. Although the apoptosis-inducing effect of GluII β inhibition appears to be p53-dependent, we found that blocking of the autophagy flux by lysosomal inhibitors enhanced the apoptotic effect of bromoconduritol independent of p53 status, thus indicating their potential combined use for cancer treatment.

Acknowledgements This study was supported by the Thailand Research Fund (grant number RSA5880016) and the CMU (Chiang Mai University) Mid-Career Research Fellowship Program. Worapong Khaodee's post-graduation study was supported by the department of Medical Technology, Chiang Mai University. We thank Dr. Tim R. Cressey, Harvard T.H. Chan School of Public Health and Chiang Mai University for his review and editing of the manuscript.

Compliance with ethical standards

Conflict of interest None declared.

References

1. B. Suradej, S. Pata, W. Kasinrerak, R. Cressey, Glucosidase II exhibits similarity to the p53 tumor suppressor in regards to structure and behavior in response to stress signals: a potential novel cancer biomarker. *Oncol. Rep.* **30**, 2511–2519 (2013)
2. S.C. Taylor, P. Thibault, D.C. Tessier, J.J. Bergeron, D.Y. Thomas, Glycopeptide specificity of the secretory protein folding sensor UDP-glucose glycoprotein:glucosyltransferase. *EMBO Rep.* **4**, 405–411 (2003)
3. J.P. Drenth, J.A. Martina, R. van de Kerkhof, J.S. Bonifacino, J.B. Jansen, Polycystic liver disease is a disorder of cotranslational protein processing. *Trends Mol. Med.* **11**, 37–42 (2005)
4. A. Li, S. Davila, L. Furu, Q. Qian, X. Tian, P.S. Kamath, B.F. King, V.E. Torres, S. Somlo, Mutations in PRKCSH cause isolated

- autosomal dominant polycystic liver disease. *Am. J. Hum. Genet.* **72**, 691–703 (2003)
5. R. Palmirotta, F. Guadagni, A. Savonarola, G. Ludovici, M.L. De Marchis, D. Palli, M. Falchetti, L. Ottini, PRKCSH GAG trinucleotide repeat is a mutational target in gastric carcinomas with high-level microsatellite instability. *Clin. Genet.* **79**, 397–398 author reply 399–400
 6. R. Forough, L. Lindner, C. Partridge, B. Jones, G. Guy, G. Clark, Elevated 80K-H protein in breast cancer: a role for FGF-1 stimulation of 80K-H. *Int. J. Biol. Markers* **18**, 89–98 (2003)
 7. K.C. Goh, Y.P. Lim, S.H. Ong, C.B. Siak, X. Cao, Y.H. Tan, G.R. Guy, Identification of p90, a prominent tyrosine-phosphorylated protein in fibroblast growth factor-stimulated cells, as 80K-H. *J. Biol. Chem.* **271**, 5832–5838 (1996)
 8. J. Yang, Y. Zhao, K. Ma, F.J. Jiang, W. Liao, P. Zhang, J. Zhou, B. Tu, L. Wang, H.H. Kampinga, Z. Xie, W.G. Zhu, Deficiency of hepatocystin induces autophagy through an mTOR-dependent pathway. *Autophagy* **7**, 748–759 (2011)
 9. J. Cui, B. Chen, H. Wang, Y. Han, X. Chen, W. Zhang, Glucosidase II beta-subunit, a novel substrate for caspase-3-like activity in rice, plays as a molecular switch between autophagy and programmed cell death. *Sci. Rep.* **6**, 31764 (2016)
 10. S. Elmore, Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* **35**, 495–516 (2007)
 11. S. Jin, Autophagy, mitochondrial quality control, and oncogenesis. *Autophagy* **2**, 80–84 (2006)
 12. S. Jin, E. White, Role of autophagy in cancer: management of metabolic stress. *Autophagy* **3**, 28–31 (2007)
 13. V. Karantza-Wadsworth, E. White, Role of autophagy in breast cancer. *Autophagy* **3**, 610–613 (2007)
 14. E. White, Deconvoluting the context-dependent role for autophagy in cancer. *Nat. Rev. Cancer* **12**, 401–410 (2012)
 15. W.J. Buchser, T.C. Laskow, P.J. Pavlik, H.M. Lin, M.T. Lotze, Cell-mediated autophagy promotes cancer cell survival. *Cancer Res.* **72**, 2970–2979 (2012)
 16. M.J. Hayat, N. Howlader, M.E. Reichman, B.K. Edwards, Cancer statistics, trends, and multiple primary cancer analyses from the Surveillance, Epidemiology, and End Results (SEER) program. *Oncologist* **12**, 20–37 (2007)
 17. J.R. Jett, D.E. Midthun, Screening for lung cancer: current status and future directions: Thomas A. Neff lecture. *Chest* **125**, 158S–162S (2004)
 18. P. Chen, J. Li, Y.C. Chen, H. Qian, Y.J. Chen, J.Y. Su, M. Wu, T. Lan, The functional status of DNA repair pathways determines the sensitization effect to cisplatin in non-small cell lung cancer cells. *Cell. Oncol.* **39**, 511–522 (2016)
 19. M. Abend, Reasons to reconsider the significance of apoptosis for cancer therapy. *Int. J. Radiat. Biol.* **79**, 927–941 (2003)
 20. A. Melet, K. Song, O. Bucur, Z. Jagani, A.R. Grassian, R. Khosravi-Far, Apoptotic pathways in tumor progression and therapy. *Adv. Exp. Med. Biol.* **615**, 47–79 (2008)
 21. S.W. Han, J. Roman, Targeting apoptotic signaling pathways in human lung cancer. *Curr. Cancer Drug Targets* **10**, 566–574 (2010)
 22. G. Cheng, D. Kong, X. Hou, B. Liang, M. He, N. Liang, S. Ma, X. Liu, The tumor suppressor, p53, contributes to radiosensitivity of lung cancer cells by regulating autophagy and apoptosis. *Cancer Biother. Radiopharm.* **28**, 153–159 (2013)
 23. Y. Takeda, K. Totani, I. Matsuo, Y. Ito, The action of bromoconduritol on ER glucosidase II. *Bioorg. Med. Chem. Lett.* **20**, 5357–5359 (2010)
 24. C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675 (2012)
 25. I. Tanida, T. Ueno, E. Kominami, LC3 and autophagy. *Methods Mol. Biol.* **445**, 77–88 (2008)
 26. M. Jung, J. Lee, H.Y. Seo, J.S. Lim, E.K. Kim, Cathepsin inhibition-induced lysosomal dysfunction enhances pancreatic beta-cell apoptosis in high glucose. *PLoS One* **10**, e0116972 (2015)
 27. R. Itotani, S. Marumo, M. Fukui, 164P: A review of prognostic factors for patients with epidermal growth factor receptor mutation positive advanced non small cell lung cancer (EGFR+NSCLC). *J. Thorac. Oncol.* **11**, S129 (2016)
 28. Y.X. Bao, X.D. Zhao, H.B. Deng, C.L. Lu, Y. Guo, X. Lu, L.L. Deng, Schedule-dependent cytotoxicity of sunitinib and TRAIL in human non-small cell lung cancer cells with or without EGFR and KRAS mutations. *Cell. Oncol.* **39**, 343–352 (2016)
 29. J.N. Contessa, M.S. Bhojani, H.H. Freeze, A. Rehemtulla, T.S. Lawrence, Inhibition of N-linked glycosylation disrupts receptor tyrosine kinase signaling in tumor cells. *Cancer Res.* **68**, 3803–3809 (2008)
 30. C. Yewale, D. Baradia, I. Vhora, S. Patil, A. Misra, Epidermal growth factor receptor targeting in cancer: a review of trends and strategies. *Biomaterials* **34**, 8690–8707 (2013)
 31. Y. Wei, Z. Zou, N. Becker, M. Anderson, R. Sumpter, G. Xiao, L. Kinch, P. Koduru, C.S. Christudass, R.W. Veltri, N.V. Grishin, M. Peyton, J. Minna, G. Bhagat, B. Levine, EGFR-mediated Beclin 1 phosphorylation in autophagy suppression, tumor progression, and tumor chemoresistance. *Cell* **154**, 1269–1284 (2013)
 32. H. Fernandes, S. Cohen, S. Bishayee, Glycosylation-induced conformational modification positively regulates receptor-receptor association: a study with an aberrant epidermal growth factor receptor (EGFRvIII/DeltaEGFR) expressed in cancer cells. *J. Biol. Chem.* **276**, 5375–5383 (2001)
 33. H.B. Guo, M. Randolph, M. Pierce, Inhibition of a specific N-glycosylation activity results in attenuation of breast carcinoma cell invasiveness-related phenotypes: inhibition of epidermal growth factor-induced dephosphorylation of focal adhesion kinase. *J. Biol. Chem.* **282**, 22150–22162 (2007)
 34. X. Wang, J. Gu, H. Ihara, E. Miyoshi, K. Honke, N. Taniguchi, Core fucosylation regulates epidermal growth factor receptor-mediated intracellular signaling. *J. Biol. Chem.* **281**, 2572–2577 (2006)
 35. A.M. Scott, F.T. Lee, N. Tebbutt, R. Herbertson, S.S. Gill, Z. Liu, E. Skrinos, C. Murone, T.H. Saunderson, B. Chappell, A.T. Papenfuss, A.M. Poon, W. Hopkins, F.E. Smyth, D. MacGregor, L.M. Cher, A.A. Jungbluth, G. Ritter, M.W. Brechbiel, R. Murphy, A.W. Burgess, E.W. Hoffinan, T.G. Johns, L.J. Old, A phase I clinical trial with monoclonal antibody ch806 targeting transitional state and mutant epidermal growth factor receptors. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 4071–4076 (2007)
 36. M. Sudo, S. Mori, V. Madan, H. Yang, G. Leong, H.P. Koeffler, Short-hairpin RNA library: identification of therapeutic partners for gefitinib-resistant non-small cell lung cancer. *Oncotarget* **6**, 814–824 (2015)
 37. G.C. Trudel, A. Herscovics, P.C. Holland, Inhibition of myoblast fusion by bromoconduritol. *Biochem. Cell Biol.* **66**, 1119–1125 (1988)
 38. K.T. Yeo, T.K. Yeo, K. Olden, Bromoconduritol treatment delays intracellular transport of secretory glycoproteins in human hepatoma cell cultures. *Biochem. Biophys. Res. Commun.* **161**, 1013–1019 (1989)
 39. G. Pulverer, J. Beuth, H.L. Ko, A. Yassin, Y. Ohshima, K. Roszkowski, G. Uhlenbruck, Glycoprotein modifications of sarcoma L-1 tumor cells by tunicamycin, swainsonine, bromoconduritol or 1-desoxynojirimycin treatment inhibits their metastatic lung colonization in Balb/c-mice. *J. Cancer Res. Clin. Oncol.* **114**, 217–220 (1988)
 40. S. Li, L. Wang, Y. Hu, R. Sheng, Autophagy regulators as potential cancer therapeutic agents: a review. *Curr. Top. Med. Chem.* **15**, 720–744 (2015)

41. V. Kaminsky, B. Zhivotovsky, Proteases in autophagy. *Biochim. Biophys. Acta* **1824**, 44–50 (2012)
42. Y.P. Yang, L.F. Hu, H.F. Zheng, C.J. Mao, W.D. Hu, K.P. Xiong, F. Wang, C.F. Liu, Application and interpretation of current autophagy inhibitors and activators. *Acta Pharmacol. Sin.* **34**, 625–635 (2013)
43. E. Kim, A. Giese, W. Deppert, Wild-type p53 in cancer cells: when a guardian turns into a blackguard. *Biochem. Pharmacol.* **77**, 11–20 (2009)
44. Z. Feng, p53 regulation of the IGF-1/AKT/mTOR pathways and the endosomal compartment. *Cold Spring Harb. Perspect. Biol.* **2**, a001057 (2010)
45. D. Crighton, S. Wilkinson, J. O'Prey, N. Syed, P. Smith, P.R. Harrison, M. Gasco, O. Garrone, T. Crook, K.M. Ryan, DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. *Cell* **126**, 121–134 (2006)
46. X. Liu, PARP inhibition as a prototype for synthetic lethal screens. *Methods Mol. Biol.* **986**, 123–137 (2013)