Bioorganic & Medicinal Chemistry 17 (2009) 7085–7092

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and discovery of pyrazole-5-carbohydrazide N-glycosides as inducer of autophagy in A549 lung cancer cells

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ARTICLE INFO

Article history:
Received 23 July 2009
Revised 2 September 2009
Accepted 3 September 2009
Available online 6 September 2009

Keywords:
Pyrazole
N-Glycoside
Synthesis
Growth inhibitory
Autophagy

ABSTRACT

A series of novel 3-aryl-1-arylmethyl-1*H*-pyrazole-5-carbohydrazide N-β-glycoside derivatives was synthesized by the reaction of substituted 1*H*-pyrazole-5-carbohydrazide with D-sugar and the effects of all the compounds on A549 cell growth were investigated. The results showed that all compounds had inhibitory effects on the growth of A549 lung cancer cells and compound **3d** possessed the highest growth inhibitory effect and induced autophagy of A549 lung cancer cells.

1. Introduction

Lung cancer is one of the leading causes of death worldwide.[1] Our understanding of the biology of cancer has undoubtedly improved in the last decade. One characteristic of cancer cells is their highly proliferative nature. Consequently, inhibition of proliferative pathways is considered to be an effective strategy to fight cancer and much attention has recently been paid to the discovery and development of new, more selective anticancer agents.[2–4]

The understanding of the mechanisms of cell-death execution and the role that they play in different diseases opens new therapeutic strategies. Induction of apoptosis by anticancer agents has been shown to correlate with tumor response. However, increasing evidence is emerging for the influence of another process that regulates the life and death of cancer cells—autophagy, an evolutionarily conserved process that involves cellular self-eating. The fact that autophagy can have both tumor suppressive and tumor promoting functions makes it an interesting topic in cancer research.[5–9] It is proposed that the role and regulation of autophagy in cancer is apparently quite complex, making autophagy a challenging, but potentially very important, target for cancer prevention and treatment.[10]

It is generally accepted that incorporation of potential biologically active moieties into body-friendly type compounds should avoid toxic or other disadvantageous side-effects and thus, obtain molecules with better chances for pharmacological applications. Sugar moieties are known to influence the pharmacokinetic properties of the respective compounds such as absorption, distribution, metabolism, and elimination. The incorporation of sugar into prospective pharmaceutical candidates is a major strategy to obtain activity and safety advantages.[11,12] A large number of bioactive natural compounds possess β-glycosidic structures such as C-glycosides,[13,14] O-glycosides,[15–22] and N-glycosides,[23–25] frequently containing one or more D-glucose, D and L-arabinose, D-xylose, and D-lyxose units.

Pyrazole derivatives have long been well known for their biological activities including such as antitumoral,[26] anti-inflammatory,[27] antinociceptive activity,[28] antimicrobial activity.[29] The synthesis of pyrazole derivatives with sugar moiety has also received considerable attention due to their broad spectrum of biological activities.[30–40] However, a search of the literature revealed that pyrazole-5-carbohydrazide derivatives with a carbohydrate moiety have not been described; therefore their synthesis and biological study seemed to be an attractive task.

Our research group is interested in the synthesis and screen of pyrazole derivatives as potential anticancer agents.[41–46] Recently, we reported the synthesis and structure–activity relationships of novel 1-arylmethyl-3-aryl-1*H*-pyrazole-5-carbohydrazide derivatives as potential agents against A549 lung cancer cells. The results showed that the carbohydrazide derivatives had more significant growth inhibitory activity against A549 cell than the carboxylate derivatives.[47–49] Thus, it is important to extend the modification of pyrazole derivatives and identify the interaction mechanism of

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doi:10.1016/j.bmc.2009.09.004

small molecules with targets. Herein, we would like to report the synthesis of 3-aryl-1-arylmethyl-1*H*-pyrazole-5-carbohydrazide with sugar moiety and the findings of their biological activities in suppressing the growth of A549 lung cancer cells by inducing autophagy.

2. Results and discussion

2.1. Synthesis of 3-aryl-1-arylmethyl-1*H*-pyrazole-5-carbohydrazide β-glycoside

The synthesis of 3-aryl-1-arylmethyl-1*H*-pyrazole-5-carbohydrazide β-glycoside derivatives has been accomplished as outlined in Scheme 1 starting from 3-aryl-1-arylmethyl-1*H*-pyrazole-5-carbohydrazide (**1**) that can be synthesized as described in our previous paper[46] and D-glucose (**2a**) or D-xylose (**2b**) in the mixture of ethanol and acetic acid over a 2–4 h reflux period in 81.2–90.5% yields.

The structures of obtained compounds were determined by IR, ^{1}H NMR and HRMS mass spectroscopy. Thus, for example 1-(4-*tert*-butylbenzyl)-3-(4-chlorophenyl)-*N*′-(3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)-1*H*-pyrazole-5-carbohydrazide (**3d**), obtained as white crystal, gave a [M+H]-ion peak at *m/z* 515.2051 in the HRESI-MS, in accord with the molecular formula $C_{26}H_{32}ClN_4O_5$. In the IR spectra, the carbonyl group absorption in hydrazide moiety was observed in the 1664 cm^{-1} and NH bands in CONH and OH absorptions were displayed in 3321–2220 cm^{-1} region, respectively. The ^{1}H NMR spectra indicated the chemical shift of the NH proton in the CONH at δ = 10.27 ppm in the form of doublet peak with coupling constant J = 5.3 Hz. Another NH proton bonded to sugar moiety appeared at 5.93 ppm as triplet peaks with coupling constant J = 4.0 Hz. The anomeric hydrogen of sugar moiety displayed at 3.84 ppm as double doublet with coupling constant J_1 = 3.1 Hz and J_2 = 8.4 Hz that is in the range resulting from an axial–axial configuration of H_1 and H_2 and therefore, for these sugar, there was a β-*N*-glycosidic linkage. The preference for formation of the β isomer can be rationalized in terms of the competition between substitution at the sterically least hindered position (β) versus substitution at a position which incurs stabilization from the anomeric effect (α). The substitution is driven predominantly by sterics and the reaction is stereoselective for the β configuration.[50] Three doublet signals of protons in hydroxy group on xylose moiety appear at 4.98 (J = 5.0 Hz), 5.06 (J = 4.5 Hz), 5.13 (J = 4.4 Hz), respectively. Five multiplet peaks attributable to 5 protons on the sugar ring were observed. The doublet of double signals appeared at δ = 5.71 with coupling constant J = 14.6 Hz and 22.5 are consistent with methylene protons in 4-*tert*-butylbenzyl moiety. Moreover, two *ortho*-aromatic protons signals in 4-*tert*-butylbenzyl moiety appeared at the range of δ = 7.19 and 7.34 ppm as doublet peaks (J = 8.2 Hz). Two *ortho*-aromatic protons signals in 4-chlorobenzene moiety appeared at the range of δ = 7.50 and 7.76 ppm as doublet peaks (J = 8.4 Hz). A singlet signal appeared at δ = 7.35 ppm are consistent with the proton in pyrazole moiety.

2.2. Effects of the compounds on the morphology of A549 cells

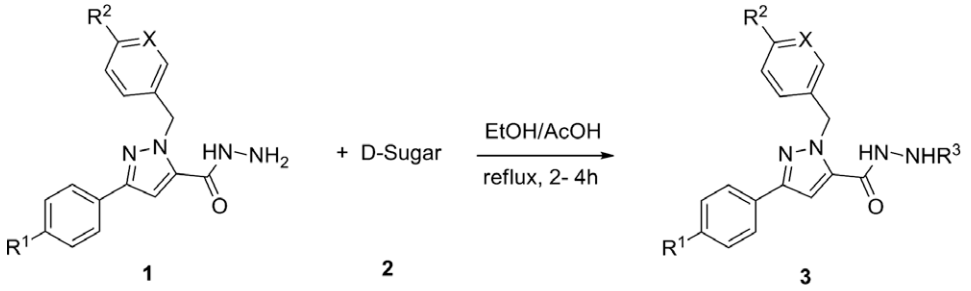
In order to evaluate the biological effects of the compounds **3a–h** on cancer cells, we used the compounds to treat A549 lung carcinoma cells at the concentrations of 10, 20 or 40 μM for 24 or 48 h. Vacuolization were observed with A549 cells treated with 40 μM **3a**, **3b**, **3e** or 20 μM **3d** at 24 h, and cell number greatly decreased in the mean time with these cells (Fig. 1). The decrease in cell number was even more obvious at 48 h for these cells. Moreover, vacuolization was observed with cells treated with 40 μM **3f**, **3g** or **3h** at 48 h as well (Fig. 2).

2.3. Inhibitory effects of compounds 3a–h on the proliferation of A549 lung cancer cells

Cell viability was analyzed by SRB assay[51,52] and the results showed that the compounds **3a–h** exhibited an inhibitory effect on the proliferation of A549 cells in a time and dose-dependent manner (Fig. 3). Among them, **3d** showed a most potent inhibitory effect on A549 cells growth, followed by **3a**, **3b**, **3e** and **3g** according to the IC_{50} values (Table 1).

2.4. Structure–activity relationships

Low IC50 values obtained for compounds **3a–g** indicated that these compounds had pronounced anti-proliferative effects on lung cancer A549 cells. From these results we could propose that the sugar moiety play more important role in molecule. For example, the IC_{50} values of compounds **3a**, **3d** and **3f** (15.17, 13.90 and 35.00, respectively) are lower than that of correspond-



1a: R^1 = H, R^2 = *t*-Bu, X = C;
1b: R^1 = Cl, R^2 = H, X = C;
1c: R^1 = Cl, R^2 = *t*-Bu, X = C;
1d: R^1 = Cl, R^2 = Cl, X = N;
1e: R^1 = OMe, R^2 = *t*-Bu, X = C;

2a: D-Glucose
2b: D-Xylose

3a: R^1 = H, R^2 = *t*-Bu, R^3 = Xylosyl, X = C;
3b: R^1 = H, R^2 = *t*-Bu, R^3 = Glucosyl, X = C;
3c: R^1 = Cl, R^2 = H, R^3 = Glucosyl, X = C;
3d: R^1 = Cl, R^2 = *t*-Bu, R^3 = Xylosyl, X = C;
3e: R^1 = Cl, R^2 = *t*-Bu, R^3 = Glucosyl, X = C;
3f: R^1 = Cl, R^2 = Cl, R^3 = Glucosyl, X = N;
3g: R^1 = OMe, R^2 = *t*-Bu, R^3 = Xylosyl, X = C;
3h: R^1 = OMe, R^2 = *t*-Bu, R^3 = Glucosyl, X = C;

Scheme 1.

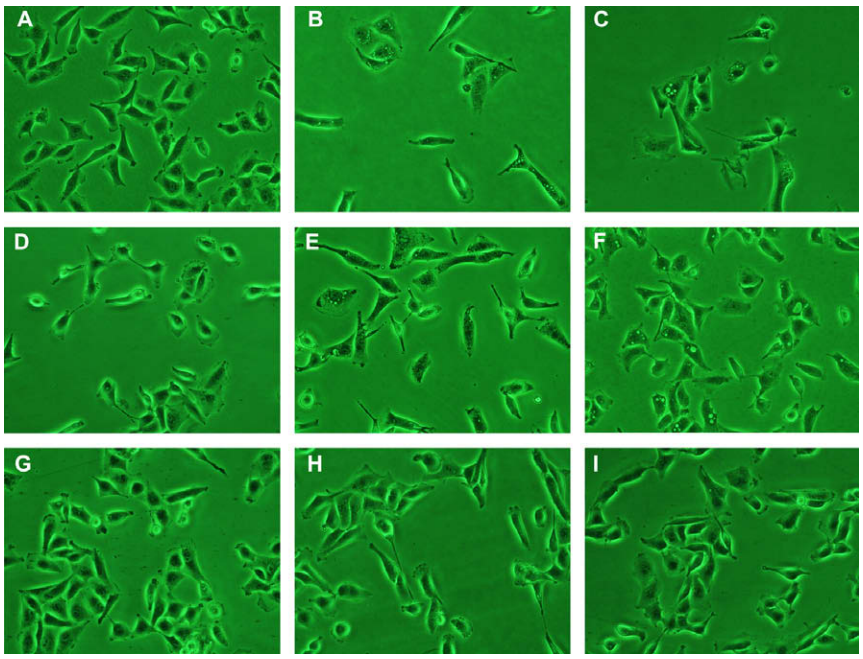


Figure 1. Effects of the compounds on A549 cell morphology at 24 h. A549 cells were treated with the 8 compounds (B–I: **3a**–**h**, respectively) or left untreated (A) for 24 h at a concentration of 40 μM, except for **3d**, which induced necrosis at 40 μM. A concentration of 20 μM was used for **3d** for all the experiments except the viability assay, for at this concentration no necrosis was detected by LDH assay. Microscopic photographs (400×) were taken under an inverted phase contrast microscope (Nikon).

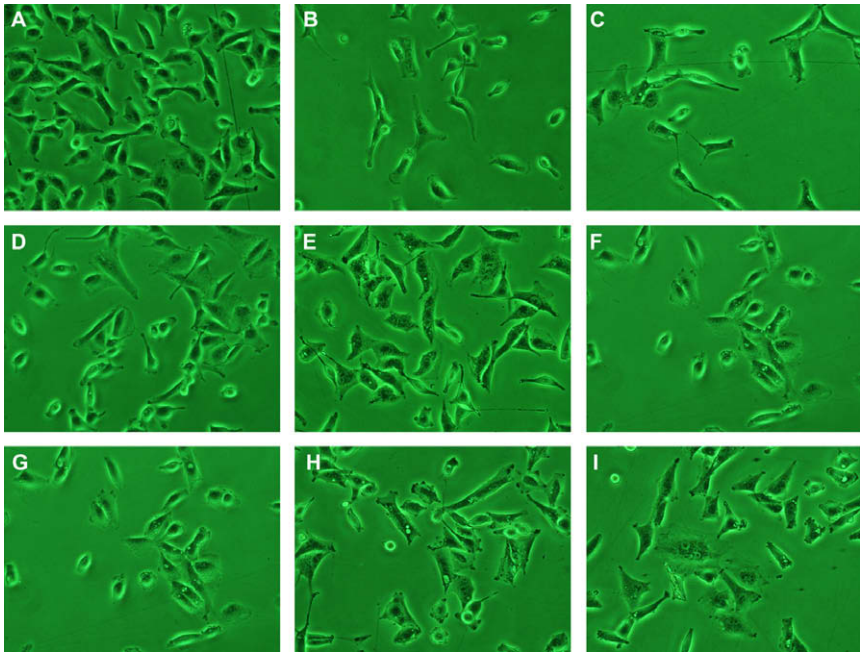


Figure 2. Effects of the compounds on A549 cell morphology at 48 h. A549 cells were treated with the 8 compounds (B–I: **3a**–**h**, respectively) or left untreated (A) for 48 h at a concentration of 40 μM, except for **3d**, for which a concentration of 20 μM was used. Microscopic photographs (400×) were taken under an inverted phase contrast microscope (Nikon).

ing compounds without sugar moiety (26.55, 18.52 and 67.71, respectively).[46] In addition, it is interesting that compounds with xylose moiety **3a**, **3d** and **3g** alter the antitumor effect significantly than that with glucose moiety **3b**, **3e** and **3h**. Also, replacement of the hydrogen at the 4-position of 3-aryl ring with electron-donating methoxy group and chlorine altered the cytotoxicities against the cancer cells tested, for example, compound **3d** with chlorine had lower IC_{50} values (IC_{50} = 13.90) than that of compound **3g** with methoxy group ((IC_{50} = 24.88). In consistent with previous observation, the substituent at the 4-position of 1-aryl ring can affect significantly activity. The compounds with a bulkier *tert*-butyl group at the 4-position of 1-aryl ring resulted in a significant activity increasing. Taken together, the cytotoxic potency was highly dependent, as expected, on the substitution types and patterns on the aryl ring as well as the kind of sugar.

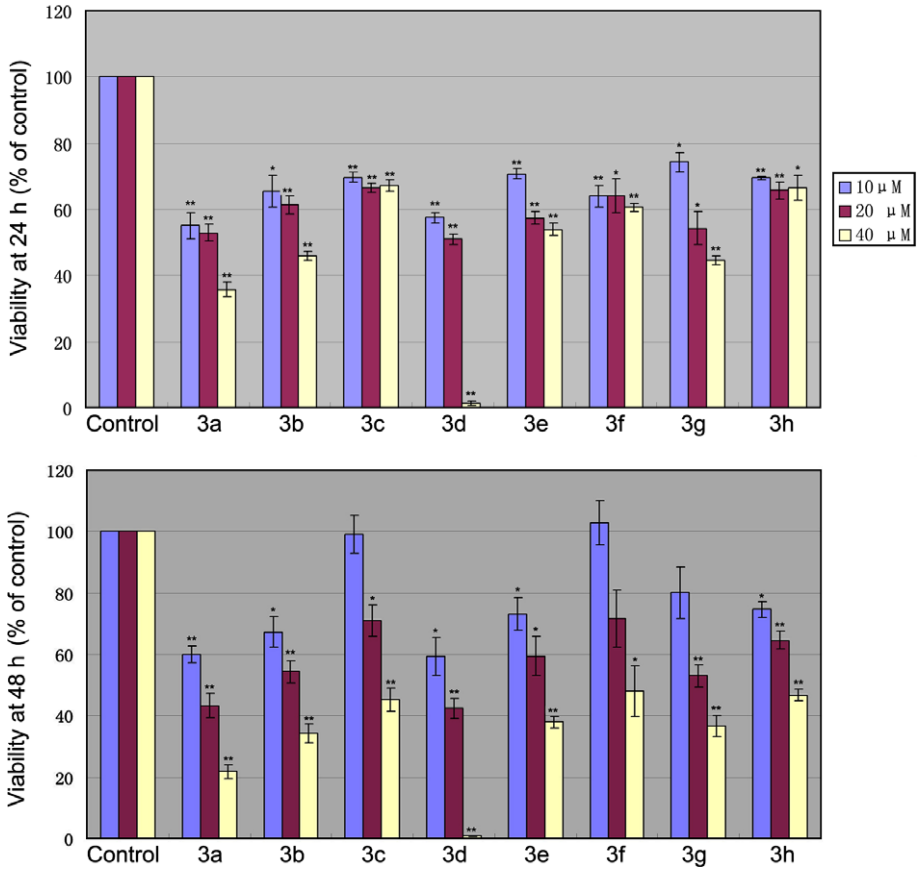


Figure 3. Effects of the compounds on A549 cell viability. A549 cells were treated with the 8 compounds or left untreated (control) for 24 h (upper) or 48 h (lower) at concentrations of 10, 20 or 40 μM. Cell viability was analyzed by SRB assay and illustrated in column figures. Results were presented as mean ± SE; $n = 3$. $^{*}P$ <0.05 versus control; $^{**}P$ <0.01 versus control.

Table 1
Growth inhibitory properties IC_{50} (μM) for the compounds at 48 h in A549 cells

Compound	3a	3b	3c	3d	3e	3f	3g	3h
IC_{50} (μM)	15.17	21.57	31.83	13.90	26.63	35.00	24.88	38.87

2.5. Compounds 3a, 3b, 3d and 3e induced autophagic cell death in A549 cells

In order to determine if the growth inhibitory effects were due to necrosis that is believed to be an unwanted side effect of cancer-fighting agents, we performed the LDH assay and the results showed that 40 μM of **3d** at 48 h caused a significant (p <0.01) increase in LDH release, indicative of necrosis induced by **3d** at this concentration. However, no increase of LDH release was detected for the other 7 compounds, at 40 μM for 48 h. We further tested if 20 μM of **3d** induced necrosis and the result showed no obvious increase of LDH release at 48 h (Fig. 4). Therefore, we used 20 μM of **3d** and 40 μM of **3a, b, c, e, f, g** and **h** for the following experiments.

The understanding of the mechanisms of cell-death execution and the role that they play in different diseases opens new therapeutic strategies. Currently, increasing evidence indicates that autophagy is a frequent cell-death mechanism and several autophagy inducers have been used as anticancer agents. Although complete tumor eradication has not been demonstrated, the antitumor effect is very promising.[53,54] Since vacuolization, a sign of autophagy, was first observed in A549 cells treated with 40 μM of **3a**, **3b**, **3e** or 20 μM of **3d**, we speculated that the inhibitory effects of these compounds on cell growth were due to autophagic cell death. It is acceptable that LC3 is the credible marker of the autophagosome in mammalian cells. Enhancement of the conversion of LC3-I to LC3-II and up-regulation of LC3 expression occurs when autophagy is induced. Thus, induction or suppression of autophagy can be easily monitored by examining the levels of LC3-II by immunoblot.[55] An alternative method for detecting the autophagic flux is measuring p62 (SQSTM1/sequestosome 1) degradation,[56] therefore we also detected the protein level of p62 in the cells. The results showed an elevated protein level of LC3-II in the A549 cells treated with these compounds at 48 h (p <0.05) though the LC3-II level was not altered at 24 h and p62 protein levels significantly (p <0.05) decreased upon drug treatment at both 24 and 48 h (Fig. 5). Taken together, these results indicated that **3a**, **3b**, **3d** and **3e** inhibit A549 cell proliferation via inducing autophagic cell death.

3. Conclusion

A dose- and time-dependent inhibition of proliferation was observed in A549 cells following compounds treatment and the induction of autophagy was demonstrated by up-regulation of LC3-II and p62 decrease. The study of structure–activity relationships showed that the cytotoxic potency of the compounds was highly dependent on the substitution types and patterns on the aryl ring as well as the kind of sugar in which compounds with xylose moiety altered the antitumor effect significantly.

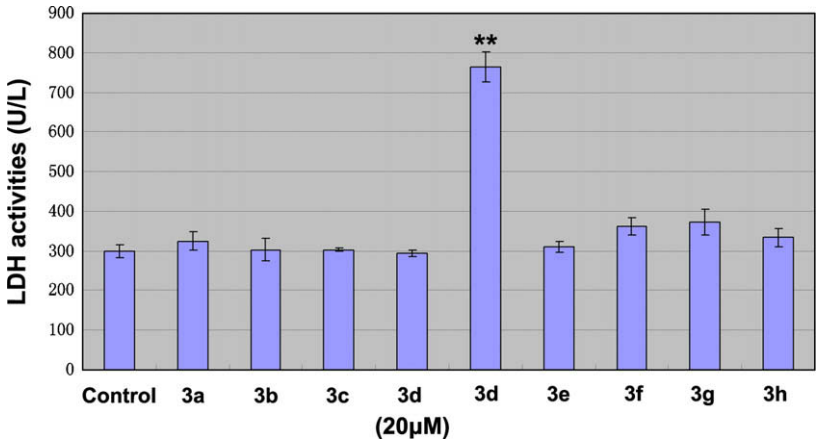


Figure 4. Effects of the compounds on LDH release in A549 cells. A549 cells were treated with the 8 compounds at the concentration of 40 μM (**3a**–**h**) or **3d** at 20 μM, or left untreated (control) for 48 h. Results were presented as mean ± SE; $n = 3$. $^{**}P < 0.01$ versus control.

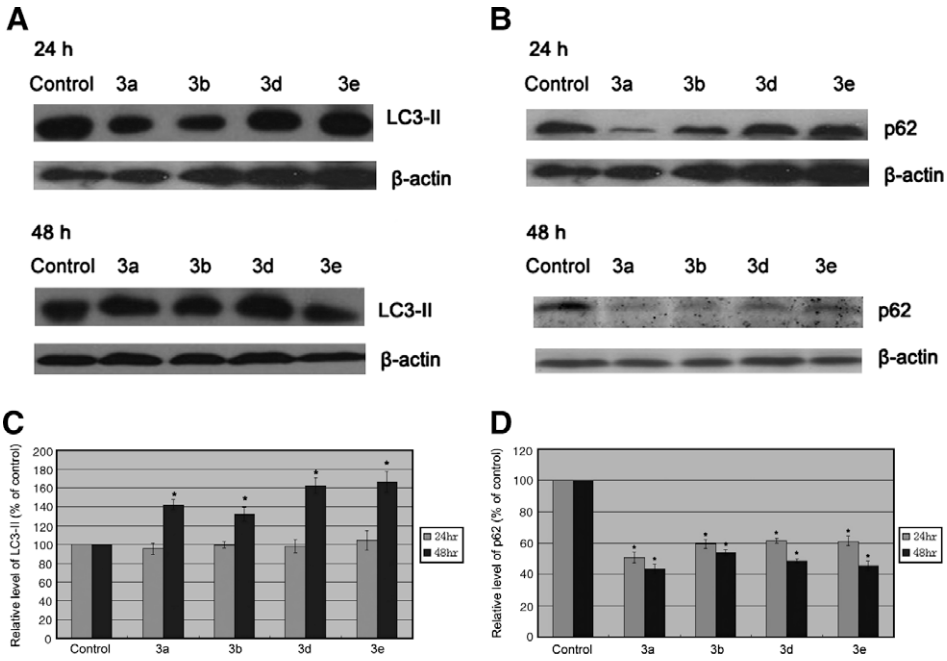


Figure 5. Western blot to test the protein level of LC3-II and p62 upon drug treatment. A549 cells were treated with 40 μM **3a**, **3b**, **3e** or 20 μM **3d** for 24 or 48 h. The protein levels of LC3-II and p62 were detected by Western blot analysis (A and B, respectively). The relative levels of LC3-II and p62 were normalized by the level of β-actin, and represented as percent of control (C and D). $^{*}P < 0.05$ vs control, $n = 3$).

4. Experimental

4.1. Reagents and apparatus

Thin-layer chromatography (TLC) was carried out on Silica Gel 60 F_{254} plates (Merck KGaA). ^{1}H NMR spectra were recorded on a Bruker Avance 400 (400 MHz) spectrometer or a Bruker Avance 300 (300 MHz) spectrometer, using DMSO as solvent and tetramethylsilane (TMS) as internal standard. Melting points were determined on an XD-4 digital micro melting point apparatus and are uncorrected. IR spectra were recorded with an IR spectrophotometer VERTEX 70 FT-IR (Bruker Optics). HRMS spectra were recorded on a Q-TOF6510 spectrograph.

RPMI 1640 was obtained from Gibco BRL Co. (Grand Island, USA). Bovine calf serum was provided by DingGuo Biotechnology (Beijing, China). Rabbit anti-human LC3b antibody (Cat. No. 2775S) was purchased from Cell Signaling (USA). Anti-p62 mouse monoclonal antibody (Cat. No. 610833) was purchased from BD Bioscience (USA). Anti-β-actin mouse monoclonal antibody (sc-47778) and HRP-conjugated goat anti-rabbit IgG (sc-2004) were obtained from Santa Cruz Biotechnology (USA). Polyclonal rabbit anti-mouse immunoglobulins/HRP (code No. P 0161) was provided by DakoCytomation (USA). SuperSignal West Pico Chemiluminescent Substrate (HRP substrate, Cat. No. 34080) was purchased from Thermo Scientific (USA). Sulforhodamine B (SRB) (Cat. No. S-9012) was provided by Sigma–Aldrich (USA).

4.2. General procedure for the synthesis of 3-aryl-1-arylmethyl-1*H*-pyrazole-5-carbohydrazide β-glycoside derivatives (3a–h)

To a suspension of glucose (**2a**) or xylose (**2b**) (0.5 mmol) in 4 mL of ethanol and one drop of glacial acetic acid was added hydrazide (**1a**–**e**) (0.5 mmol). The reaction mixture was stirred and heated at reflux for 2–4 h. After cooling to room temperature,

the precipitate was filtered to afford the corresponding product **3a**–**h** in 81.2–90.5% yields.

4.2.1. 1-(4-*tert*-Butylbenzyl)-3-phenyl-*N*′-(3,4,5-trihydroxy-tetrahydro-2*H*-pyran-2-yl)-1*H*-pyrazole-5-carbohydrazide (3a)

Yield: 85.8%, White solid, mp: 192–195 °C; IR (KBr) *v*: 3435, 3269 (–OH, –NH), 1664 (C=O) cm^{-1}; ^1H NMR (400 MHz, DMSO) *δ:* 1.24 (s, 9H, $C(CH_3)_3$), 3.00–3.07 (m, 2H, CH_2), 3.16–3.22 (m, 1H, CH), 3.23–3.29 (m, 1H, CH), 3.72 (dd, 1H, J_1 = 11.2 Hz, J_2 = 5.1 Hz, CH), 3.85 (dd, 1H, J_1 = 8.4 Hz, J_2 = 3.3 Hz, CH), 4.94 (d, 1H, *J* = 4.8 Hz, OH), 5.01 (d, 1H, *J* = 4.6 Hz, OH), 5.10 (d, 1H, *J* = 4.4 Hz, OH), 5.71 (dd, 2H, J_1 = 21.2 Hz, J_2 = 14.6 Hz, NCH_2Ar), 5.92 (t, 1H, *J* = 4.1 Hz, NH), 7.19 (d, 2H, *J* = 8.2 Hz, ArH), 7.33 (d, 4H, *J* = 7.2 Hz, ArH, pyrazolyl-H), 7.43 (t, 2H, *J* = 7.5 Hz, ArH), 7.75 (d, 2H, *J* = 7.5 Hz, ArH), 10.23 (d, 1H, *J* = 5.3 Hz, NHC=O); HRMS *m/z* $[M+H]^+$ calcd 481.2445, found 481.2428.

4.2.2. 1-(4-*tert*-Butylbenzyl)-3-phenyl-*N*′-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)-1*H*-pyrazole-5-carbohydrazide (3b)

Yield: 81.2%, White solid, mp: 227–229 °C; IR (KBr) *v*: 3446, 3384, 3345, 3271 (–OH, –NH), 1662 (C=O) cm^{-1}; ^1H NMR (400 MHz, DMSO) *δ:* 1.23 (s, 9H, $C(CH_3)_3$), 2.98–3.07 (m, 2H, CH_2), 3.12–3.15 (m, 1H, CH), 3.21–3.26 (m, 1H, CH), 3.43–3.49 (m, 1H, CH), 3.67–3.71 (m, 1H, CH), 3.88 (dd, 1H, J_1 = 8.7 Hz, J_2 = 2.8 Hz, CH), 4.32 (t, 1H, *J* = 5.6 Hz, 6-OH), 4.96 (d, 1H, *J* = 5.3 Hz, OH), 5.05 (d, 1H, *J* = 4.7 Hz, OH), 5.15 (d, 1H, *J* = 4.2 Hz, OH), 5.71 (dd, 2H, J_1 = 28.5 Hz, J_2 = 14.6 Hz, NCH_2Ar), 5.95 (t, 1H, *J* = 3.7 Hz, NH), 7.20 (d, 2H, *J* = 8.2 Hz, ArH), 7.32–7.36 (m, 4H, ArH, pyrazolyl-H), 7.43 (t, 2H, *J* = 7.6 Hz, ArH), 7.76 (d, 2H, *J* = 7.4 Hz, ArH), 10.17 (d, 1H, *J* = 5.0 Hz, NHC=O); HRMS *m/z* $[M+H]^+$ calcd 511.2551, found 511.2549.

4.2.3. 1-Benzyl-3-(4-chlorophenyl)-*N*′-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)-1*H*-pyrazole-5-carbohydrazide (3c)

Yield: 90.1%, White solid, mp: 224–228 °C; IR (KBr) *v*: 3444, 3388, 3299, 3266 (–OH, –NH), 1665 (C=O) cm^{-1}; ^1H NMR (400 MHz, DMSO) *δ:* 3.02–3.07 (m, 2H, CH_2), 3.11–3.15 (m, 1H, CH), 3.20–3.26 (m, 1H, CH), 3.43–3.49 (m, 1H, CH), 3.67–3.72 (m, 1H, CH), 3.87 (dd, 1H, J_1 = 8.7 Hz, J_2 = 2.9 Hz, CH), 4.27 (t, 1H, *J* = 5.7 Hz, 6-OH), 4.91 (d, 1H, *J* = 5.2 Hz, OH), 4.97 (d, 1H, *J* = 4.8 Hz, OH), 5.08 (d, 1H, *J* = 4.3 Hz, OH), 5.75 (dd, 2H, J_1 = 26.4 Hz, J_2 = 14.7 Hz, NCH_2Ar), 5.92 (t, 1H, *J* = 3.9 Hz, NH), 7.26 (t, 3H, *J* = 7.0 Hz, ArH), 7.33 (t, 2H, *J* = 7.4 Hz, ArH), 7.37 (s, 1H, pyrazolyl-H), 7.50 (d, 2H, *J* = 8.4 Hz, ArH), 7.78 (d, 2H, *J* = 8.6 Hz, ArH), 10.15 (d, 1H, *J* = 5.0 Hz, NHC=O); HRMS *m/z* $[M+H]^+$ calcd 489.1535, found 489.1519.

4.2.4. 1-(4-*tert*-Butylbenzyl)-3-(4-chlorophenyl)-*N*′-(3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)-1*H*-pyrazole-5-carbohydrazide (3d)

Yield: 81.6%, White solid, mp: 192–194 °C; IR (KBr) *v*: 3445, 3321, 3280, 3220 (–OH, –NH), 1664 (C=O) cm^{-1}; ^1H NMR (400 MHz, DMSO) *δ:* 1.23 (s, 9H, $C(CH_3)_3$), 3.00–3.06 (m, 2H, CH_2), 3.16–3.21 (m, 1H, CH), 3.25–3.30 (m, 1H, CH), 3.71 (dd, 1H, J_1 = 11.2 Hz, J_2 = 5.1 Hz, CH), 3.84 (dd, 1H, J_1 = 8.4 Hz, J_2 = 3.1 Hz, CH), 4.98 (d, 1H, *J* = 5.0 Hz, OH), 5.06 (d, 1H, *J* = 4.5 Hz, OH), 5.13 (d, 1H, *J* = 4.4 Hz, OH), 5.71 (dd, 2H, J_1 = 22.5 Hz, J_2 = 14.6 Hz, NCH_2Ar), 5.94 (t, 1H, *J* = 4.1 Hz, NH), 7.19 (d, 2H, *J* = 8.2 Hz, ArH), 7.34 (d, 2H, *J* = 8.2 Hz, ArH), 7.35 (s, 1H, pyrazolyl-H), 7.50 (d, 2H, *J* = 8.4 Hz, ArH), 7.76 (d, 2H, *J* = 8.4 Hz, ArH), 10.27 (d, 1H, *J* = 5.3 Hz, NHC=O); HRMS *m/z* $[M+H]^+$ calcd 515.2061, found 515.2051.

4.2.5. 1-(4-*tert*-Butylbenzyl)-3-(4-chlorophenyl)-*N*′-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)-1*H*-pyrazole-5-carbohydrazide (3e)

Yield: 86.3%, White solid, mp: 205–207 °C; IR (KBr)*v*: 3488, 3438, 3286 (–OH, –NH), 1668 (C=O) cm^{-1}; ^1H-NMR (300 MHz, DMSO) *δ:* 1.23 (s, 9H, $C(CH_3)_3$), 2.93–3.07 (m, 2H, CH_2), 3.11–3.15 (m, 1H, CH), 3.19–3.27 (m, 1H, CH), 3.41–3.49 (m, 1H, CH), 3.66–3.71 (m, 1H, CH), 3.87 (dd, 1H, J_1 = 8.7 Hz, J_2 = 3.0 Hz, CH), 4.34 (t, 1H, *J* = 5.7 Hz, 6-OH), 4.97 (d, 1H, *J* = 5.1 Hz, OH), 5.07 (d, 1H, *J* = 4.5 Hz, OH), 5.16 (d, 1H, *J* = 4.2 Hz, OH), 5.70 (dd, 2H, J_1 = 24.6 Hz, J_2 = 15.3 Hz, NCH_2Ar), 5.95 (t, 1H, *J* = 3.8 Hz, NH), 7.20 (d, 2H, *J* = 8.4 Hz, ArH), 7.35 (d, 2H, *J* = 8.1 Hz, ArH), 7.35 (s, 1H, pyrazolyl-H), 7.50 (d, 2H, *J* = 8.4 Hz, ArH), 7.77 (d, 2H, *J* = 8.4 Hz, ArH), 10.19 (d, 1H, *J* = 4.8 Hz, NHC=O); HRMS *m/z* $[M+H]^+$ calcd 545.2161, found 545.2169.

4.2.6. 3-(4-Chlorophenyl)-1-((6-chloropyridin-3-yl)methyl)-*N*′-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)-1*H*-pyrazole-5-carbohydrazide (3f)

Yield: 90.5%, White solid, mp: 223–224 °C; IR (KBr) *v*: 3445, 3381, 3313 (–OH, –NH), 1664 (C=O) cm^{-1}; ^1H NMR (400 MHz, DMSO) *δ:* 3.00–3.08 (m, 2H, CH_2), 3.11–3.13 (m, 1H, CH), 3.19–3.25 (m, 1H, CH), 3.41–3.49 (m, 1H, CH), 3.67–3.71 (m, 1H, CH), 3.88 (dd, 1H, J_1 = 8.5 Hz, J_2 = 2.5 Hz, CH), 4.27 (t, 1H, *J* = 5.6 Hz, 6-OH), 4.90 (d, 1H, *J* = 5.2 Hz, OH), 4.96 (d, 1H, *J* = 4.6 Hz, OH), 5.08 (d, 1H, *J* = 4.3 Hz, OH), 5.77 (dd, 2H, J_1 = 20.9 Hz, J_2 = 15.0 Hz, NCH_2Ar), 5.92 (t, 1H, *J* = 3.8 Hz, NH), 7.40 (s, 1H, pyrazolyl-H), 7.49 (d, 1H, *J* = 8.2 Hz, ArH), 7.50 (d, 2H, *J* = 8.5 Hz, ArH), 7.71 (dd, 1H, J_1 = 8.3 Hz, J_2 = 2.3 Hz, ArH), 7.77 (d, 2H, *J* = 8.5 Hz, ArH), 8.36 (d, 1H, *J* = 2.0 Hz, ArH), 10.21 (d, 1H, *J* = 5.1 Hz, NHC=O); HRMS *m/z* $[M+H]^+$ calcd 524.1098, found 524.1095.

4.2.7. 1-(4-*tert*-Butylbenzyl)-3-(4-methoxyphenyl)-*N*′-(3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)-1*H*-pyrazole-5-carbohydrazide (3g)

Yield: 90.2%, White solid, mp: 200–201 °C; IR (KBr) *v*: 3451, 3296, 3238 (–OH, –NH), 1665 (C=O) cm^{-1}; ^1H NMR (300 MHz, DMSO) *δ:* 1.23 (s, 9H, $C(CH_3)_3$), 2.98–3.07 (m, 2H, CH_2), 3.15–3.32 (m, 2H, CH), 3.71 (dd, 1H, J_1 = 11.0 Hz, J_2 = 5.0 Hz, CH), 3.78 (s, 3H, OCH_3), 3.84 (dd, 1H, J_1 = 8.6 Hz, J_2 = 3.2 Hz, CH), 5.01 (d, 1H, *J* = 4.8 Hz, OH), 5.10 (d, 1H, *J* = 4.5 Hz, OH), 5.17 (d, 1H, *J* = 4.2 Hz, OH), 5.69 (dd, 2H, J_1 = 19.8 Hz, J_2 = 14.7 Hz, NCH_2Ar), 5.94 (t, 1H, *J* = 4.2 Hz, NH), 6.99 (d, 2H, *J* = 8.7 Hz, ArH), 7.18 (d, 2H, *J* = 8.4 Hz, ArH), 7.26 (s, 1H, pyrazolyl-H), 7.34 (d, 2H, *J* = 8.1 Hz, ArH), 7.67 (d, 2H, *J* = 9.0 Hz, ArH), 10.25 (d, 1H, *J* = 5.1 Hz, NHC=O); HRMS *m/z* $[M+H]^+$ calcd 511.2551, found 511.2543.

4.2.8. 1-(4-*tert*-Butylbenzyl)-3-(4-methoxyphenyl)-*N*′-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)-1*H*-pyrazole-5-carbohydrazide (3h)

Yield: 90%, White solid, mp: 215–216 °C; IR (KBr) *v*: 3429, 3288 (–OH, –NH), 1667 (C=O) cm^{-1}; ^1H NMR (400 MHz, DMSO) *δ:* 1.23 (s, 9H, $C(CH_3)_3$), 2.98–3.07 (m, 2H, CH_2), 3.11–3.15 (m, 1H, CH), 3.20–3.26 (m, 1H, CH), 3.43–3.49 (m, 1H, CH), 3.66–3.71 (m, 1H, CH), 3.78 (s, 3H, OCH_3), 3.87 (dd, 1H, J_1 = 8.7 Hz, J_2 = 2.9 Hz, CH), 4.26 (t, 1H, *J* = 5.6 Hz, 6-OH), 4.90 (d, 1H, *J* = 5.2 Hz, OH), 4.97 (d, 1H, *J* = 4.8 Hz, OH), 5.10 (d, 1H, *J* = 4.2 Hz, OH), 5.67 (dd, 2H, J_1 = 26.7 Hz, J_2 = 14.6 Hz, NCH_2Ar), 5.91 (t, 1H, *J* = 3.9 Hz, NH), 6.99 (d, 2H, *J* = 8.8 Hz, ArH), 7.19 (d, 2H, *J* = 8.4 Hz, ArH), 7.24 (s, 1H, pyrazolyl-H), 7.34 (d, 2H, *J* = 8.4 Hz, ArH), 7.68 (d, 2H, *J* = 8.7 Hz, ArH), 10.09 (d, 1H, *J* = 5.0 Hz, NHC=O); HRMS *m/z* $[M+H]^+$ calcd 541.2657, found 541.2669.

4.3. Cell culture

A549 lung cancer cells were cultured in RPMI 1640 medium at 37 °C with 5% CO_2, and 95% air, supplemented with 10% (v/v) bovine calf serum and 80 U/ml gentamicin. The cells were seeded onto 96-well plates or other appropriate dishes containing the medium at the density of 6250/cm^2.

4.4. Cell viability assay

Cells were seeded in 96-well plates. After 24 h, cells were treated with 0.1% DMSO (as control) or the compounds at indicated concentrations for specified time durations. Cell viability was evaluated by sulforhodamine B (SRB) assay.[51,52] Briefly, pour off the medium and fix cells by adding 100 μL of cold 10% trichloroacetic acid (TCA) and incubate for 1 h at 4 °C. Discard the supernatant and then wash the plates five times with deionized water. Add 50 μL of 0.4% (W/V) SRB solution in 1% acetic acid to each well and shake 5 min on titer plate shaker. Wash the plates five times with 1% acetic acid and subsequently add 100 μL of 10 mM unbuffered Tris base (pH 10.5) to dissolve the bound dye. Mix 5 min on a microtiter plate shaker and read optical densities at the wavelength of 510 nm using SpectraMAX 190 microplate spectrophotometer (GMI Co., USA).

4.5. LDH assay

Cell culture medium was collected after 48 h treatment with 0.1% DMSO (as control) or the compound. LDH assay was performed using Lactate Dehydrogenase (LDH) kit (Nanjing Jiancheng Co., China) according to the manufacturer's instructions.

4.6. Western blot analysis

Cells were washed twice with ice cold PBS and were then lysed in protein lysis buffer (0.5% SDS in 25 mM Tris–HCl, pH 7.5, 4 mM EDTA, 100 mM NaCl, 1 mM PMSF, 10 μg/mL leupeptin and 10 μg/mL soybean trypsin inhibitor). The protein concentration of the cells was determined by the Bradford method.[57] After adding loading buffer and boiling, equal amount of protein was loaded onto a 15% SDS polyacrylamide gel. After electrophoresis, the resolved protein was electrophoretically transferred to a Polyvinylidene Fluoride (PVDF) membrane (Millipore, MA, USA). The membrane was blocked with 5% non-fat milk in PBST (PBS containing 0.05% Tween 20) for 1 h at room temperature. Subsequently the membrane was probed with Rabbit anti-human LC3b antibody, anti-p62 mouse monoclonal antibody or anti-β-actin mouse monoclonal antibody overnight at 4 °C, and was then washed twice with PBST, each time for 5 min. The membrane was subsequently incubated with HRP-conjugated goat anti-rabbit IgG or polyclonal rabbit anti-mouse immunoglobulins/HRP for 1 h at room temperature and then washed three times with PBST. Then the membrane was incubated with HRP substrate for 5 min and the fluorescence signal were detected with X-ray films. Intensity of the protein bands was quantified using Quantity-One software (Bio-Rad).

4.7. Statistical analysis

Data were presented as means ± SE from at least three independent experiments and analyzed by Student's *t*-test. Differences at $p < 0.05$ were considered statistically significant.

Acknowledgments

This study was supported by the Science and Technology Developmental Project of Shandong Province (2008GG10002034 and 2007GG20002004) and National Natural Science Foundation of China (90813022).

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