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Synthesis and discovery of autophagy inducers for A549 and H460 lung cancer cells, novel 1-(2′-hydroxy-3′-aroxypropyl)-3-aryl-1*H*-pyrazole-5-carbohydrazide derivatives

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ABSTRACT

A series of novel 1-(2′-hydroxy-3′-aroxypropyl)-3-aryl-1*H*-pyrazole-5-carbohydrazide derivatives were synthesized, and the effects of the compounds on A549 cell growth were investigated. The results showed that all of the 1-(2′-hydroxy-3′-aroxypropyl)-3-aryl-1*H*-pyrazole-5-carbohydrazide derivatives **2** could inhibit the growth of A549 cells in dosage- and time-dependent manners. Typically, compound **2a** and **2d** induced A549 cells to autophagy but did not cause apoptosis and necrosis in the cells, and **2d** had the most autophagy inducing effect in H460 cells. More importantly, **2a** and **2d** did not inhibit the growth of HUVEC cells.

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Cancer is the second leading cause of death in developed countries, accounting for nearly one in five deaths. Lung cancer is one of the leading causes of death in the world. Since current treatment modalities are inadequate, novel therapies are needed to reduce the effects of the increasing incidence in pulmonary neoplasm.[1,2] An elaborate search of new anticancer agents has primarily been triggered by the unveiling of new molecular targets on which they intervene, followed by the discovery of novel classes of compounds that interact with such targets.[3,4] In our effort to discover and develop apoptosis inducers as potential new anticancer agents, we have identified several classes of molecules as novel apoptosis inducers, including safrole oxide, 1-alkoxy-3-(3′,4′-methylenedioxy)phenyl-2-propanol, morpholinone derivatives, and 2,3-dihydro-3-hydroxymethyl-1,4-benzoxazine derivatives.[5–17]

Induction of apoptosis by anticancer agents has been shown to correlate with tumor response; however, non-apoptotic forms of cell death, such as autophagy and extrinsic senescence, have also been shown to contribute to the overall tumor response. Non-apoptotic forms of programmed cell death are targets for novel approaches in anticancer therapy. Autophagy is a process in which subcellular membranes undergo dynamic morphological changes that lead to the degradation of cellular proteins and cytoplasmic organelles. Recently, interest in autophagy has been renewed among oncologists, because different types of cancer cells undergo autophagy after various anticancer therapies.[18–22]

Many pyrazole derivatives are known to exhibit a wide range of biological properties such as anti-hyperglycemic, analgesic, anti-inflammatory, anti-pyretic, anti-bacterial, hypoglycemic, sedative–hypnotic activity, and anticoagulant activity.[23–25] Extensive studies have been devoted to arylpyrazole derivatives such as Celecoxib, a well-known cyclooxygenase-2 inhibitor.[26–28] In the previous paper, we investigated the effects of ethyl 1-(2′-hydroxy-3′-aroxypropyl)-3-aryl-1*H*-pyrazole-5-carboxylate derivatives on the growth of A549 lung cancer cell and found that these compounds could suppress A549 lung cancer cell growth.[29] Recently, we described the synthesis and preliminary biological evaluation of ethyl 1-arylmethyl-3-aryl-1*H*-pyrazole-5-carboxylate derivatives.[30] The modification of pyrazole such as basic substituent moiety should provide potential bioactivities. A few of heterocyclic carbohydrazide derivatives have been reported.[31] 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.[32]

Thus, it is important to expand the modification of pyrazole derivatives and identify the interaction mechanism of small molecules with targets. Herein, we would like to report the synthe-

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sis of 1-(2′-hydroxy-3′-aroxypropyl)-3-aryl-1*H*-pyrazole-5-carbohydrazide and the findings of their biological activities in suppressing the growth of A549 lung cancer cells by inducing autophagy.

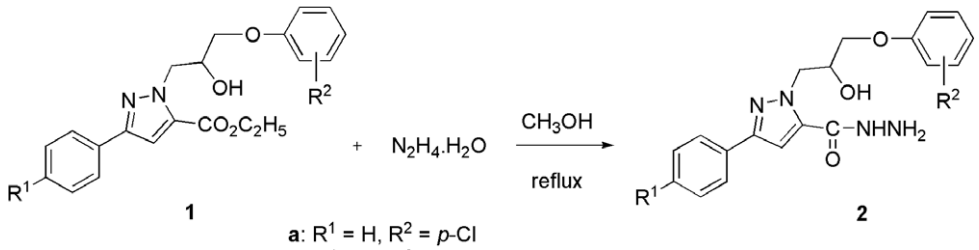
Chemistry. The synthesis of 1-(2′-hydroxy-3′-aroxypropyl)-3-aryl-1*H*-pyrazole-5-carbohydrazide derivatives has been accomplished as outlined in Scheme 1 starting from ethyl 1-(2′-hydroxy-3′-aroxypropyl)-3-aryl-1*H*-pyrazole-5-carboxylate (**1**) that can be synthesized as described in our previous paper.[29] For example, 1-(3′-(4′-chlorophenoxy)-2-hydroxypropyl)-3-(4′-chlorophenyl)-1*H*-pyrazole-5-carbohydrazide (**2d**) was synthesized in 86% yield by the reaction of ethyl 1-(3′-(4′-chlorophenoxy)-2-hydroxypropyl)-3-(4′-chlorophenyl)-1*H*-pyrazole-5-carboxylate with hydrazine hydrate in methanol over a 4 h reflux period. The structures of **2a**–**2h** were determined by IR, ^{1}H NMR and mass spectroscopy.[33] Thus, for example, **2d**, obtained as white crystal, gave a [M+H]-ion peak at *m*/*z* 422.4 in the ESI-MS, in accord with the molecular formula $C_{19}H_{18}Cl_2N_4O_3$. The carbonyl group absorption in hydrazide moiety was observed in the 1678 cm^{-1} in IR spectra. The ^{1}H NMR (DMSO) spectra indicated two *ortho*-aromatic protons signals in *p*-Cl phenol moiety appeared at the range of δ = 6.87 and 7.27 ppm as doublet peaks (J = 9.0 Hz). Two *ortho*-aromatic protons signals in *p*-Cl phenyl moiety appeared at the range of δ = 7.52 and 7.63 ppm as doublet peaks (J = 8.4 Hz). One proton signal in pyrazole moiety appeared at δ = 6.85 ppm. Two double doublet signals appeared at δ = 3.92 and 4.03 ppm are consistent with two methylene protons, respectively. Three protons appeared at the range of 4.26–4.37 ppm as multilet peaks. The signals of hydroxyl and NH appeared at δ = 5.55 and 9.98 ppm, respectively. The element analysis shows satisfactory accord.

Effects of the compounds on the viability of A549 lung cancer cells and HUVEC cells. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay is widely accepted as a reliable way to measure the cell proliferation rate, and conversely when metabolic events lead to apoptosis or necrosis. The data obtained by MTT assay showed that compounds **2a–2h** had inhibitory effects on the growth of A549 cells in dosage- and time-dependent manners as indicated by the results shown in Figure 1. As typically shown in Figure 1, exposure of cells to **2a**, **2d**, **2e**, and **2g** at 40 μM for 24 h resulted in cell viability decrease from 100% to 72–62% ($p < 0.05$). When the exposure continued on to 48 h, compared with the control group, the cell viability reduced more significantly from 100% to 56–40% ($p < 0.01$). Further, exposure of cells to **2a**, **2d**, and **2e** at 80 μM for 48 and 96 h, the cell viability reduced more significantly from 100% to 29% and 6%, respectively ($p < 0.01$). As shown in Figure 1B, **2a** and **2d** did not inhibit the growth of HUVEC (human umbilical vein endothelial cells), as normal somatic cells, for 24 h and 48 h. They might not influence the normal somatic cell growth and have no toxicity to normal cells. Interestingly, compound **2d** at 10 μM promotes HUVEC cell growth at 24 h. Taken together, compound **2d** was the most effective compound in suppressing A549 cell growth. The growth inhibitory properties (IC_{50}) for the compounds **2a**–**2h** are listed in Table 1.

Effects of the compounds on the morphology of A549 cells. Morphological changes are associated with the physiological and pathological processes in A549 cells. After A549 cells were treated with compounds **2a** and **2d** at 10–80 μM for 24 and 48 h, the cells vacuolated gradually as the increase of the concentration and the prolongation of the time. Compounds **2b**, **2c**, and **2e**–**2h** had a similar but inferior vacuolating effect in A549 cells compared to **2a** and **2d** (data not shown). The obviously morphological changes of the cells treated with compounds **2a** and **2d** at 80 μM for 24 and 48 h were observed under a contrast phase microscope, as shown in Figure 2. Such morphological changes were not apparent in the control cells. The results showed that the autophagy should take place. It was confirmed by the analysis of nuclear and acidic compartment morphology as well as LDH activity assay.

Effect of the compounds on nuclear and acidic compartment morphology. DNA fragmentation, chromatin condensation, cell shrinkage, and membrane blebbing are the characteristics of apoptotic cells. To detect whether compounds induce apoptosis in A549 and H460 lung cancer cells, we performed acridine orange (AO) staining under an Olympus BH-2 fluorescence microscope. The morphology revealed the appearance of large membranous vacuoles in the cytoplasm, but the nuclei of these cells appeared normal with minimum evidence of chromatin condensation. The formation of these membranous vacuoles increased progressively with increasing compound exposure time (Fig. 3). The results suggested that the compounds did not induce apoptosis in A549 and H460 cells. Autophagy is the process of sequestering cytoplasmic proteins into the lytic compartment of the cell, and it is characterized by the formation of acidic vesicular organelles. In acridine orange-stained cells, the cytoplasm and nucleolus fluoresce bright green, whereas acidic compartments fluoresce bright red. Therefore, acridine orange-staining has been accepted as a marker for autophagy.[20,34] Thus, the results showed there were increased acidic vacuoles in the cells treated with compounds **2a** and **2d** compared to the untreated cells. Other compounds had a similar but inferior effect compared to **2a** and **2d** (data not shown). Such morphological changes were not apparent in the control cells. Therefore, the compounds **2a** and **2d** might induce autophagy in A549 and H460 cells.

Effect of the compounds on LDH release in A549 cells. To detect whether compounds resulted in necrosis of A549 cells, LDH activ-



a: R^1 = H, R^2 = *p*-Cl
b: R^1 = H, R^2 = *p*-NO_2
c: R^1 = H, R^2 = *o*-OCH_3
d: R^1 = *p*-Cl, R^2 = *p*-Cl
e: R^1 = *p*-Cl, R^2 = *p*-NO_2
f: R^1 = *p*-Cl, R^2 = *o*-OCH_3
g: R^1 = *p*-OCH_3, R^2 = *p*-Cl
h: R^1 = *p*-OCH_3, R^2 = *o*-OCH_3

Scheme 1. Synthesis of 1-(2′-hydroxy-3′-aroxypropyl)-3-aryl-1*H*-pyrazole-5-carbohydrazide.

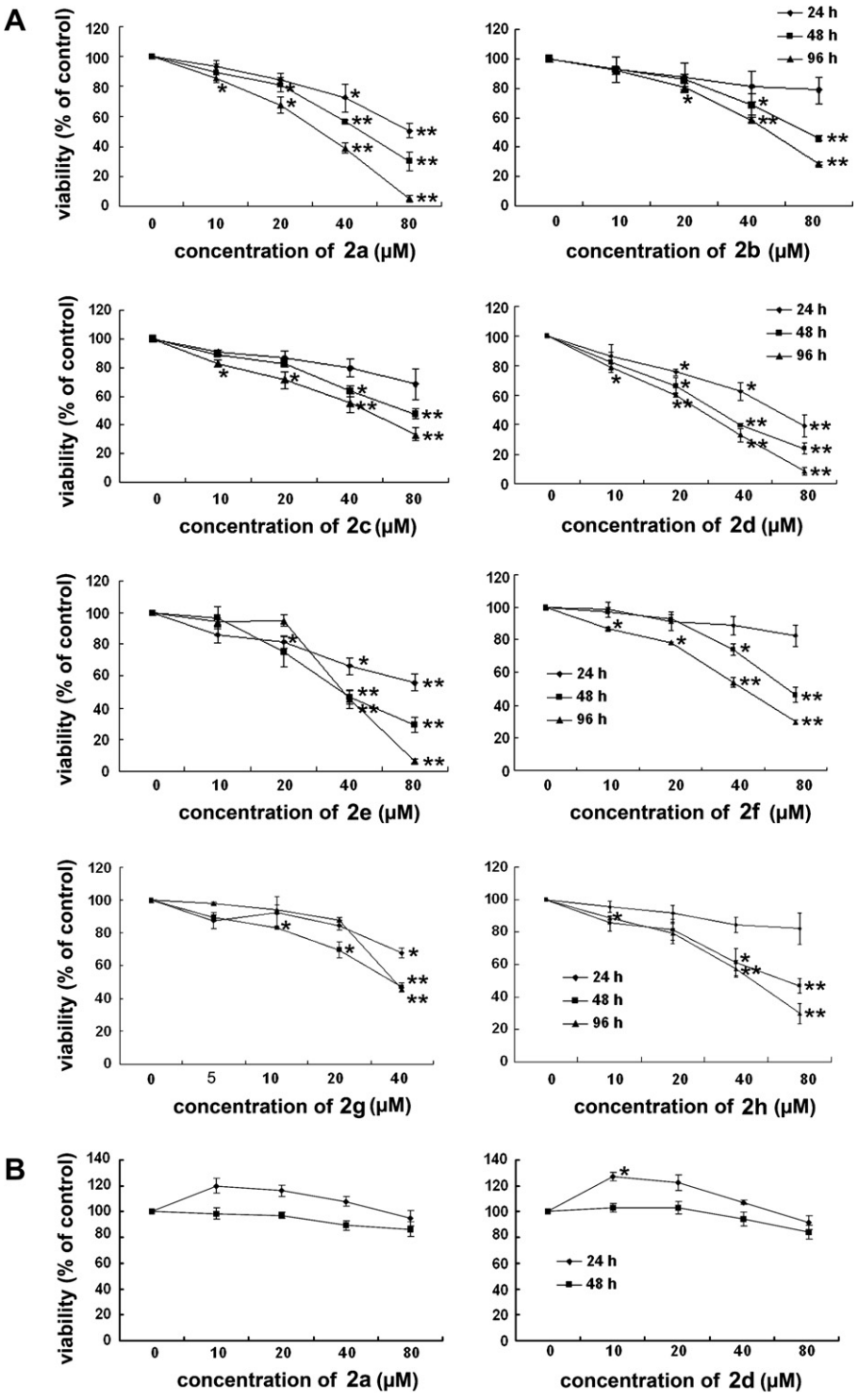


Figure 1. Effects of the compounds on the viability of A549 lung cancer and HUVEC cells. (A) Effects of the compounds **2a**–**2h** on the viability of A549 lung cancer cells. The viability of the cells treated with the compounds at the concentrations indicated for 24, 48, and 96 h, respectively. (B) Effects of compounds **2a** and **2d** on the viability of HUVEC cells for 24 and 48 h. Data are means ± SE from three independent experiments. ($^{*}P < 0.05$ and $^{**}P < 0.01$ vs the DMSO group.)

Table 1
Growth inhibitory properties (IC_{50}) for the compounds **2a**–**h** at 48 and 96 h

Compound	**2a**	**2b**	**2c**	**2d**	**2e**	**2f**	**2g**	**2h**
96 h (μM)	30	45	49	27	37	40	35	51
48 h (μM)	48	72	72	32	39	74	39	70

ity in cell culture medium was measured. As shown in Figure 4, there was no significant difference ($p > 0.05$) in LDH release between the cells of control group and the cells treated with the compounds at 80 μM for 24 and 48 h. The results indicated that the compounds at the test range of concentration did not cause necrosis in A549 cells (Fig. 4) and in H460 cells (data not shown). Thus, our results suggested that the compounds **2a–2h** induced A549 and H460 cells to autophagy, among them **2a** and **2d** had more effect in A549 cells, and **2d** had the most effect in H460 cells.

In summary, all of the 1-(2′-hydroxy-3′-aroxypropyl)-3-aryl-1*H*-pyrazole-5-carbohydrazide derivatives **2** could inhibit the

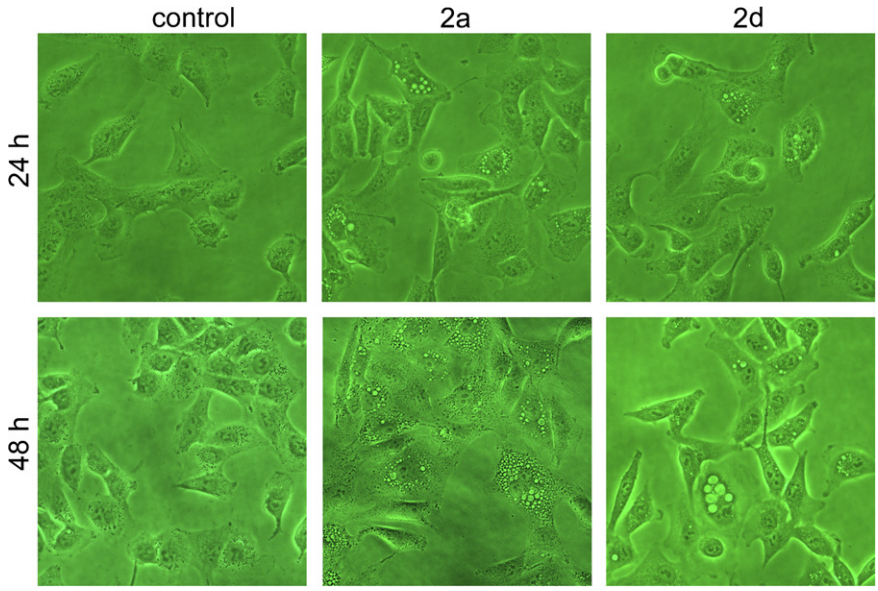


Figure 2. Morphology image of A549 cells treated with the compounds **2a** and **2d** (80 μM) for 24 and 48 h. Control: the cells treated with DMSO 0.1% (v/v) as a vehicle control (600×).

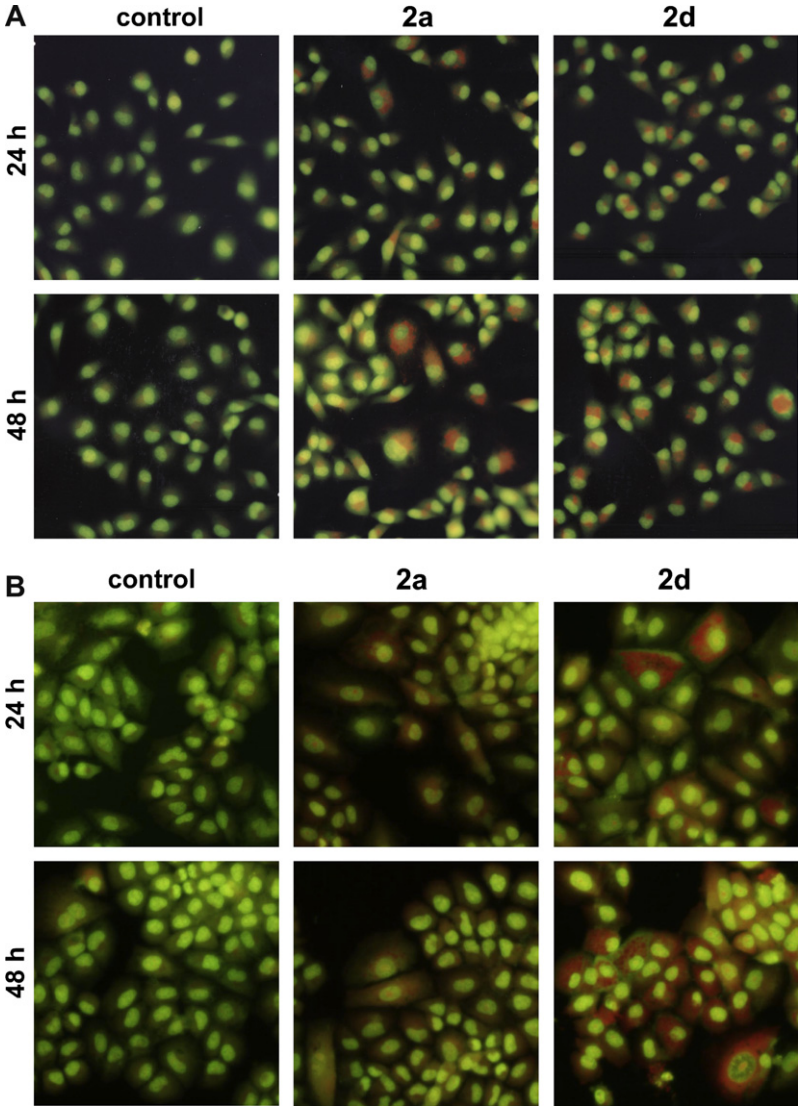


Figure 3. Determination of autophagy in A549 (A) and H460 (B) cells by acridine orange-staining. Control: the cells treated with DMSO 0.1% (v/v) as a vehicle control; **2a** and **2d**: the cells treated with the compounds **2a** and **2d** (80 μM) for 24 and 48 h, respectively (400×).

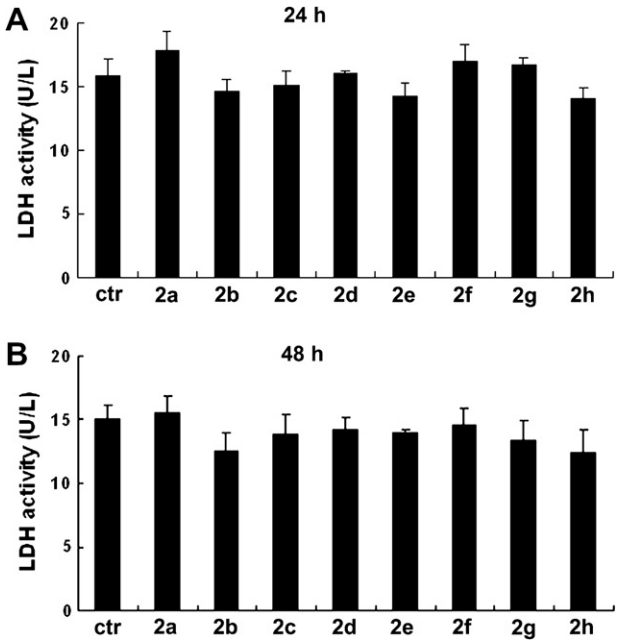


Figure 4. Effects of compounds **2a**–**2h** on LDH activities of A549 cells. The culture medium was collected as samples for LDH assay after 24 and 48 h treatment at the concentration of 80 μM. ($p > 0.05$ vs control group, $n = 3$.)

growth of A549 cells in dosage- and time-dependent manners, typically compounds **2a** and **2d** induced A549 cells to autophagy but did not cause apoptosis and necrosis in the cells, and **2d** had the most autophagy inducing effect in H460 cells. More importantly, **2a** and **2d** did not inhibit the growth of HUVEC cells.

Acknowledgment

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33. Spectral data of compounds **2a**–**2h**.
1-(3′-(4′-*Chlorophenoxy*)-2′-*hydroxypropyl*)-3-*phenyl*-1*H*-*pyrazole*-5-*carbohydrazide* (**2a**): Yield 58%, white solid. mp 148–150 °C; IR (KBr) *v*: 3476–3219 (OH, NH, NH_2), 1675 (C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ: 3.80 (dd, 1H, $J = 6.3$, 9.6 Hz), 3.93 (dd, 1H, $J = 4.5$, 9.6 Hz), 4.08 (br s, 2H, NH_2), 4.31 (dd, 1H, $J = 7.2$, 13.8 Hz), 4.37 (dd, 1H, $J = 4.2$, 13.8 Hz), 4.43–4.46 (m, 1H), 4.92 (br s, 1H, OH), 6.69 (d, 2H, $J = 6.9$ Hz), 6.81 (s, 1H), 7.20 (d, 2H, $J = 6.9$ Hz), 7.39–7.45 (m, 5H, ArH), 8.75 (br s, 1H, NH); ESI-MS: 387.4 $(M+H)^+$.
1-(2′-*Hydroxy*-3′-(4′-*nitrophenoxy*)*propyl*)-3-*phenyl*-1*H*-*pyrazole*-5-*carbohydrazide* (**2b**): Yield 56%, white solid. mp 171–174 °C; IR (KBr) *v*: 3450–3286 (OH, NH, NH_2), 1670 (C=O), 1517 and 1254 (NO_2) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$/DMSO-d_6) δ: 4.02–4.03 (m, 2H, CH_2), 4.30–4.41 (m, 2H, CH_2), 4.43–4.51 (m, 1H, CH), 5.53 (br s, 1H, OH), 6.76 (s, 1H), 6.91 (d, 2H, $J = 9.3$ Hz), 7.42–7.46 (m, 5H, ArH), 8.15 (d, 2H, $J = 9.3$ Hz), 9.18 (br s, 1H, NH); ESI-MS: 398.4 $(M+H)^+$.
1-(2′-*Hydroxy*-3′-(2′-*methoxyphenoxy*)*propyl*)-3-*phenyl*-1*H*-*pyrazole*-5-*carbohydrazide* (**2c**): Yield 55%, oil. IR (film) *v*: 3492–3180 (OH, NH, NH_2), 1681 (C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ: 3.77 (s, 3H, OCH_3), 3.93–4.04 (m, 2H, CH_2), 4.40–4.41 (m, 2H, CH_2), 4.46–4.52 (m, 1H, CH), 6.82–7.00 (m, 5H, ArH and pyrazole moiety), 7.42 (m, 5H, ArH), 9.46 (br s, 1H, NH); ESI-MS: 383.5 $(M+H)^+$.1-(3′-(4′-*Chlorophenoxy*)-2′-*hydroxypropyl*)-3-(4′-*chlorophenyl*)-1*H*-*pyrazole*-5-*carbo hydrazide* (**2d**): Yield 86%, white solid. mp 166–168 °C; IR (KBr) *v*: 3353–3090 (NH, NH_2), 1678 (C=O) cm^{-1}; ^1H NMR (300 MHz, DMSO) δ: 3.92 (dd, 1H, $J = 4.5$, 13.9 Hz, CH_2), 4.03 (dd, 1H, $J = 6.9$, 13.9 Hz, CH_2), 4.26-4.37 (m, 3H, CH_2, CH), 5.55 (d, 1H, $J = 4.8$ Hz, OH), 6.85 (s, 1H), 6.87 (d, 2H, $J = 9.0$ Hz, ArH), 7.27 (d, 2H, $J = 9.0$ Hz, ArH), 7.52 (d, 2H, $J = 8.4$ Hz, ArH), 7.63 (d, 2H, $J = 8.4$ Hz, ArH), 9.98 (br s, 1H, NH); ESI-MS: 422.4 $(M+H)^+$.
3-(4′-*Chlorophenyl*)-1-(2′-*hydroxy*-3′-(4′-*nitrophenoxy*)*propyl*)-1*H*-*pyrazole*-5-*carbohydrazide* (**2e**): Yield 87%, pale yellowish solid. mp 156–159 °C; IR (KBr) *v*: 3421–3122 (OH, NH, NH_2), 1673 (C=O), 1517 and 1254 (NO_2) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ: 3.90–4.18 (m, 4H, NH_2 and CH_2), 4.30–4.40 (m, 2H, CH_2), 4.45–4.51 (m, 1H, CH), 5.31 (br s, 1H, OH), 6.80 (s, 1H), 6.87 (d, 2H, $J = 9.0$ Hz), 7.40–7.47 (m, 5H, ArH), 8.18 (d, 2H, $J = 9.0$ Hz, ArH), 8.80 (br s, 1H, NH); ESI-MS: 432.5 $(M+H)^+$.
3-(4′-*Chlorophenyl*)-1-(2′-*hydroxy*-3′-(2′-*methoxyphenoxy*)*propyl*)-1*H*-*pyrazole*-5-*carbohydrazide* (**2f**): Yield 93%, white solid, mp 150–153 °C; IR (KBr) *v*: 3391–3350 (OH, NH, NH_2), 1681 (C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ: 3.77 (s, 3H, CH_3), 3.91 (dd, 1H, $J = 5.6$, 9.8 Hz, CH_2), 3.99 (dd, 1H, $J = 5.1$, 9.8 Hz, CH_2), 4.36 (d, 2H, $J = 5.6$ Hz, CH_2), 4.48–4.53 (m, 1H, CH), 6.80–6.91 (m, 4H, ArH), 6.94–6.98 (m, 1H, ArH), 7.33–7.37 (m, 4H, ArH); ESI-MS: 417.5 $(M+H)^+$.
1-(3′-(4′-*Chlorophenoxy*)-2′-*hydroxypropyl*)-3-(4′-*methoxyphenyl*)-1*H*-*pyrazole*-5-*carbohydrazide* (**2g**): Yield 81%, white solid, mp 180–182 °C; IR (KBr) *v*: 3348–2935 (OH, NH, NH_2), 1680 (C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ: 3.79 (dd, 1H, $J = 5.6$, 9.6 Hz, CH_2), 3.83 (s, 3H, CH_3), 3.90 (dd, 1H, $J = 4.4$, 9.6 Hz, CH_2), 4.34–4.45 (m, 3H, CH and CH_2), 6.67 (d, 2H, $J = 7.0$ Hz, ArH), 6.88 (d, 2H, $J = 6.0$ Hz, ArH), 6.92 (s, 1H, ArH), 7.19 (d, 2H, $J = 6.0$ Hz, ArH), 7.28 (d, 2H, $J = 7.0$ Hz, ArH), 9.39 (br s, 1H, NH); ESI-MS: 417.5 $(M+H)^+$.
1-(2′-*Hydroxy*-3′-(2′-*methoxyphenoxy*)*propyl*)-3-(4′-*methoxyphenyl*)-1*H*-*pyrazole*-5-*carbohydrazide* (**2h**): Yield 71%, white oil. IR (film) *v*: 3350–2933 (OH, NH, NH_2), 1677 (C=O) cm^{-1}; ^1H NMR (300 MHz, DMSO/$CDCl_3$) δ: 3.71 (s, 3H, CH_3),3.80 (s, 3H, CH_3), 3.99 (d, 1H, $J = 4.8$ Hz, NH_2), 4.15–4.42 (m, 2H, CH_2), 4.43–4.50 (m, 3H, CH and CH_2), 5.42 (d, 1H, $J = 4.8$ Hz, OH), 6.67 (s, 1H, ArH), 6.82-6.96 (m, 4H, ArH), 7.03 (d, 2H, $J = 8.7$ Hz, ArH), 7.51 (d, 2H, $J = 8.7$ Hz, ArH), 9.41 (s, 1H, NH); ESI-MS: 413.6 $(M+H)^+$.
34. Paglin, S.; Hollister, T.; Delohery, T.; Hackett, N.; McMahill, M.; Sphicas, E.; Domingo, D.; Yahalom, J. *Cancer Res.* **2001**, *61*, 439.