Delineation of the Role of Glycosylation in the Cytotoxic Properties of Quercetin using Novel Assays in Living Vertebrates

Si-Hwan Park, [†,∥] Hyun Jung Kim,[‡,∥] Soon-Ho Yim,[§] Ah-Ra Kim,[†] Nisha Tyagi,[†] Haihong Shen,[†] Kyung Keun Kim,[⊥] Boo Ahn Shin,[⊥] Da-Woon Jung,*[,†] and Darren R. Williams*[,†]

[†]School of Life Sciences, Gwangju Institute of Science and Technology, Gwangju 500-712, Republic of Korea

[‡]College of Pharmacy and Natural Medicine Research Institute, Mokpo National University, Jeonnam 534-729, Republic of Korea

[§] College of Public Health and Welfare, Dongshin University, Jeonnam 520-714, Republic of Korea

[⊥]Chonnam National University Medical School, Gwangju 501-746, Republic of Korea

Supporting information

1. General experimental procedures

Conventional ^{1}H, ^{13}C NMR, HSQC, and HMBC spectra were recorded using a Varian Unity INOVA 500 spectrometer in MeOH-d_4, and chemical shifts are expressed in δ (ppm) and the coupling constants are in Hz. ESIMS was performed on a Varian 320-MS TQ mass spectrometer connected to a Varian Prostar HPLC system (Palo Alto, CA). Column chromatography was carried out on silica gel 60 (70-230 mesh) (Merck, Darmstadt, Germany) and Sephadex LH-20 (GE Healthcare, Uppsala, Sweden). HPLC separations were carried out with an Agilent HP1100 series system (Santa Clara, CA) which comprises a degasser, two binary mixing pumps, a column oven and a diode array detector. A Waters SunFire™ C18 (5 μm, 4.6×150 mm) (Milford, MA) and a SunFire™ Prep C18 (5 μm, 10×150 mm), a SunFire™ Prep C18 OBD (5 μm, 19×150 mm) HPLC columns were used for analytical and semi-preparative HPLC analysis. UV absorptions were monitored at the wavelengths of 254, 280 and 350 nm.

2. Plant materials

The flowers of *H. japonica* were collected at Arboretum of Korea Expressway Corporation in Jeonju, Jeonbuk Province, Republic of Korea in March 2009. The plant materials were identified by Prof. Hyun Jung Kim, one of the authors, and a voucher specimen (No. P2009HJ) was deposited at the herbarium in the College of Pharmacy, Mokpo National University, Republic of Korea.

3. Extraction and isolation of quercetin glycosides (2–5)

The shade-dried flowers of *H. japonica* (338.5 g) were powdered and extracted with MeOH (1.5 L

× 3) at room temperature. After filtration, the MeOH extract was evaporated under reduced pressure to obtain a dark brown residue (77.1 g). The concentrated MeOH extract was suspended in water and partitioned with *n*-hexane and EtOAc and *n*-BuOH. Isolation of quercetin glycosides from the EtOAc extract was performed as follows. The EtOAc extract (14.3 g) was subjected to Sephadex LH-20 chromatography on elution with MeOH, and silica gel column chromatography with a $CHCl_3$/MeOH step-gradient system (91:9 → 25:75) to afford 12 fractions. Each fraction was screened by analytical HPLC mode in order to trace flavonoid compounds. As a result, flavonoid-containing fraction 7 (2.59 g) was separated by HPLC using the gradient eluent system with acetonitrile and water containing 0.1% formic acid, i.e. 12% acetonitrile to 35% acetonitrile for 30 min. Amounts of the isolated compounds (**2**–**5**) were as follows; **2** (t_R 14.62 min; 16.7 mg), **3** (t_R 14.93 min; 34.6 mg), **4** (t_R 17.86 min; 33.9 mg), and **5** (t_R 18.91 min; 103.3 mg).

4. Spectroscopic data for quercetin glycosides (2–5)

Hyperoside (Quercetin 3-*O*-β-galactopyranoside) (**2**): yellow amorphous powder; UV λ_{max} 267, 295, 356 nm; ^{1}H NMR (MeOH-d_4, 500 MHz) δ 7.85 (1H, brs, H-2′), 7.58 (1H, brd, 8.0, H-6′), 6.86 (1H, d, 8.0, H-5′), 6.40 (1H, brs, H-8), 6.20 (1H, brs, H-6), 5.16 (1H, d, 7.5, H-1′′), 3.85 (1H, m, H-3′′), 3.82 (1H, m, H-4′′), 3.65(1H, m, H-6′′), 3.56 (2H, m, H-2′′,6′′), 3.48 (1H, m, H-5′′); ^{13}C NMR (MeOH-d_4, 125 MHz) δ 179.7 (C-4), 166.4 (C-7), 163.1 (C-5), 158.9 (C-2), 158.6 (C-8a), 150.1 (C-4′), 146.0 (C-3′), 135.9 (C-3), 123.1 (C-6′), 123.0 (C-1′), 117.9 (C-5′), 116.2 (C-2′), 105.7 (C-4a), 105.6 (C-1′′), 100.1 (C-8), 95.0 (C-6), 77.3 (C-5′′), 75.2 (C-3′′), 73.3 (C-2′′), 70.2 (C-4′′), 62.1 (C-

6′′); ESIMS *m/z* 463 $[M–H]^-$ ($C_{21}H_{20}O_{12}$)

Isoquercitrin (Quercetin 3-*O*-β-glucopyranoside) (**3**): yellow amorphous powder; UV λ_{max} 257, 303, 354 nm; ^{1}H NMR (MeOH-d_4, 500 MHz) δ 7.71 (1H, brs, H-2′), 7.58 (1H, brd, 8.5, H-6′), 6.86 (1H, d, 8.5, H-5′), 6.38 (1H, s, H-8), 6.19 (1H, s, H-6), 5.25 (1H, d, 7.5, H-1′′), 3.72 (1H, dd, 12.0, 2.0, H-6′′), 3.58 (1H, dd, 12.0, 5.5, H-6′′), 3.49 (1H, t, 8.0, H-2′′), 3.44 (1H, t, 9.0, H-3′′), 3.35 (1H, t, 9.0, H-4′′), 3.24 (1H, m, H-5′′); ^{13}C NMR (MeOH-d_4, 125 MHz) δ 179.6 (C-4), 166.3 (C-7), 163.2 (C-5), 159.1 (C-8a), 158.6 (C-2), 150.0 (C-4′), 146.0 (C-3′), 135.7 (C-3), 123.3 (C-6′), 123.2 (C-1′), 117.7 (C-5′), 116.1 (C-2′), 105.8 (C-4a), 104.4 (C-1′′), 100.1 (C-8), 94.9 (C-6), 78.5 (C-5′′), 78.2 (C-3′′), 75.9 (C-2′′), 71.3 (C-4′′), 62.7 (C-6′′); ESIMS *m/z* 463 $[M–H]^-$ ($C_{21}H_{20}O_{12}$)

Quercitrin (Quercetin 3-*O*-α-rhamnopyranoside) (**4**): brown-yellow amorphous powder; UV λ_{max} 257, 347 nm; ^{1}H NMR (MeOH-d_4, 500 MHz) δ 7.34 (1H, brs, H-2′), 7.30 (1H, brd, 8.0, H-6′), 6.91 (1H, d, 8.0, H-5′), 6.36 (1H, s, H-8), 6.19 (1H, s, H-6), 5.35 (1H, s, H-1′′), 4.23 (1H, s, H-2′′), 3.76 (1H, brd, 7.5, H-3′′), 3.42 (1H, m, 8, H-5′′), 3.34 (1H, d, 9.5, H-4′′), 0.94 (3H, d, 6.5, H-6′′); ^{13}C NMR (MeOH-d_4, 125 MHz) δ 179.7 (C-4), 166.0 (C-7), 163.3 (C-5), 159.4 (C-2), 158.6 (C-8a), 149.9 (C-4′), 146.5 (C-3′), 136.4(C-3), 123.1 (C-6′), 123.0 (C-1′), 117.7 (C-5′), 116.5 (C-2′), 106.0 (C-4a), 103.7 (C-1′′), 100.0 (C-8), 94.9 (C-6), 73.4 (C-4′′), 72.2 (C-3′′), 72.2 (C-2′′), 72.0 (C-5′′), 17.8 (C-6′′); ESIMS *m/z* 447 $[M–H]^-$ ($C_{21}H_{20}O_{11}$)

Spiraeoside (Quercetin 4′-*O*-β-glucopyranoside) (**5**): yellow amorphous powder; UV λ_{max} 254, 266, 367 nm; ^{1}H NMR (MeOH-d_4, 500 MHz) δ 7.74 (1H, brs, H-2′), 7.69 (1H, brd, 8.5, H-6′), 7.28 (1H, d, 8.5, H-5′), 6.37 (1H, s, H-8), 6.17 (1H, s, H-6), 4.91 (1H, d, 7.5, H-1′′), 3.94 (1H, dd, 12.0, 1.5, H-

6′′), 3.74 (1H, dd, 12.0, 5.5, H-6′′), 3.55 (1H, t, 8.0, H-2′′), 3.52 (1H, t, 10.0, H-3′′), 3.49 (1H, m, H-5′′), 3.44 (1H, t, 9.5, H-4′′); ^{13}C NMR (MeOH-d_4, 125 MHz) δ 177.6 (C-4), 165.9 (C-7), 162.6 (C-5), 158.4 (C-8a), 148.2 (C-4′), 148.0 (C-3′), 147.0 (C-2), 138.0 (C-3), 123.3 (C-1′), 121.4 (C-6′), 117.7 (C-5′), 116.6 (C-2′), 104.7 (C-4a), 103.5 (C-1′′), 99.5 (C-8), 94.6 (C-6), 78.5 (C-5′′), 77.7(C-3′′), 75.0 (C-2′′), 71.4 (C-4′′), 62.6 (C-6′′); ESIMS *m/z* 463 $[M-H]^-$ ($C_{21}H_{20}O_{12}$)

5. References

(1) Zhou, T.; Chen, B.; Fan, G.; Chai, Y.; Wu, Y. *J. Chromatogr. A* **2006**, *1116*, 97-101.

(2) Williams, C. A.; Harborne, J. B. In *The Flavonoids*, Harborne, J. B., Ed.; Chapman &Hall: London, UK, 1994: pp 337-385.

(3) Lee, J. H.; Ku, C. H.; Baek, N.-I.; Kim, S.-H.; Park, H. W.; Kim, D. K. *Arch. Pharm. Res.* **2004**, *27*, 40-43.

(4) Kim, Y.-K.; Kim, Y. S.; Choi, S. U.; Ryu, S. Y. *Arch. Pharm. Res.* **2004**, *27*, 44-47.

(5) Park, Y.-K.; Lee, C. Y. **1996**, *44*, 34-36.

Figure S1. HPLC profile for quercetin glycosides (**2**–**5**) from *H. japonica*. HPLC chromatographic profile and UV spectra of phenolic compounds **2**-**5**. The fraction was separated by HPLC using the gradient eluent system, acetonitrile and water containing 0.1% formic acid, i.e. 12% acetonitrile to 35% acetonitrile, with a Waters SunFire™ (4.6 × 150 mm, 5 μm) column for 30 min at the flow rate of 1 mL/min.

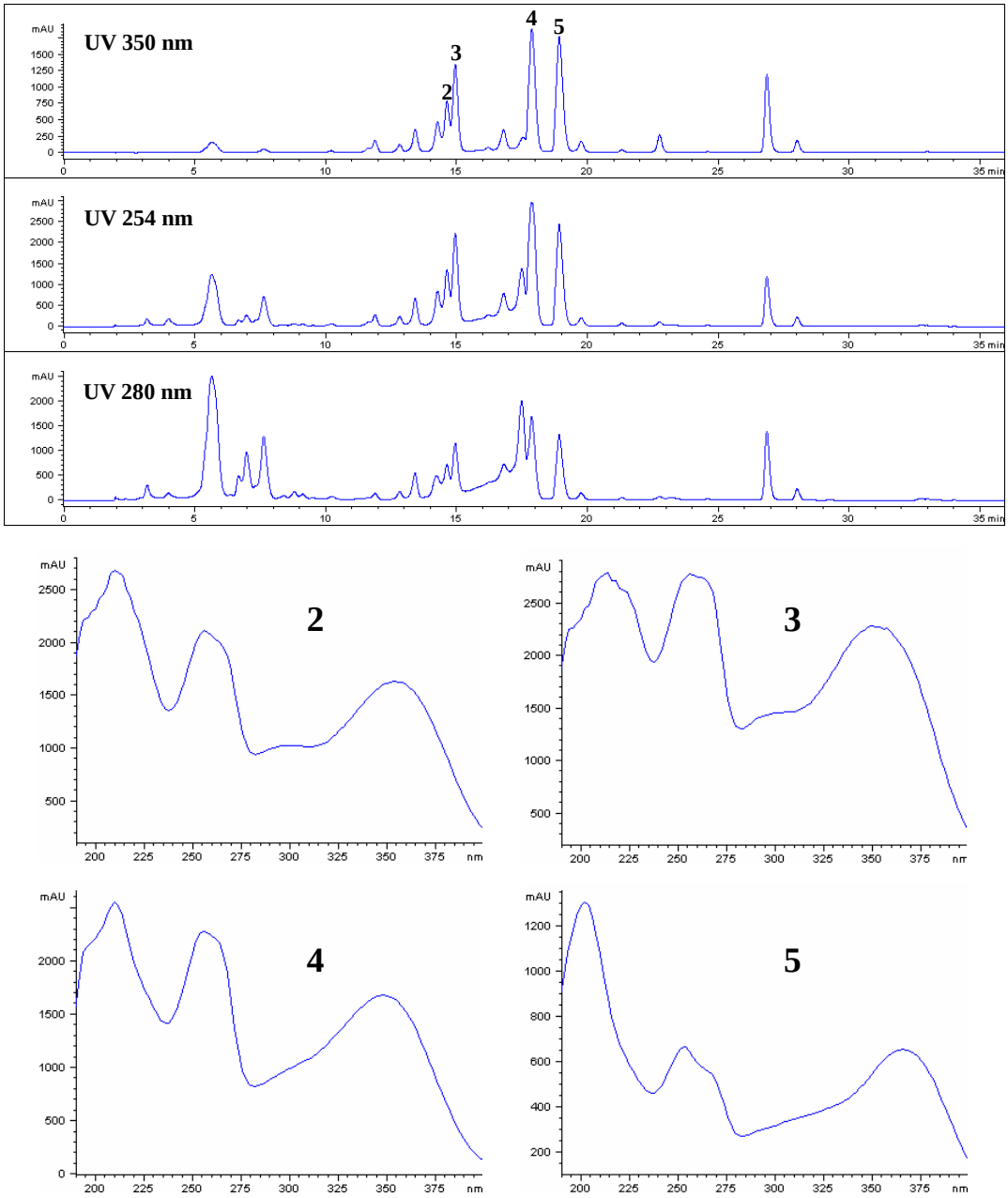

Figure S2. Antitumor activity of quercetin 3-*O*-galactoside (**2**), quercetin 3-*O*-glucoside (**3**), and quercetin 3-*O*-rhamnoside (**4**) in vivo. A) Representative phase contrast, fluorescent and merged images of HCT116 cancer cells extracted from enzymatically dissociated embryos. Xenografted embryos were treated with 80 μM of **2**, **3**, or **4** for 4 days (6 animals/treatment). Arrows indicate HCT116 cancer cell. B) Inhibition of cancer cell proliferation by **2**, **3**, or **4** in vivo. DMSO and 10 μM API-H7 were used as negative and positive controls, respectively. Error=SEM (Standard error of the mean); *=p<0.05 compared to the negative control group.

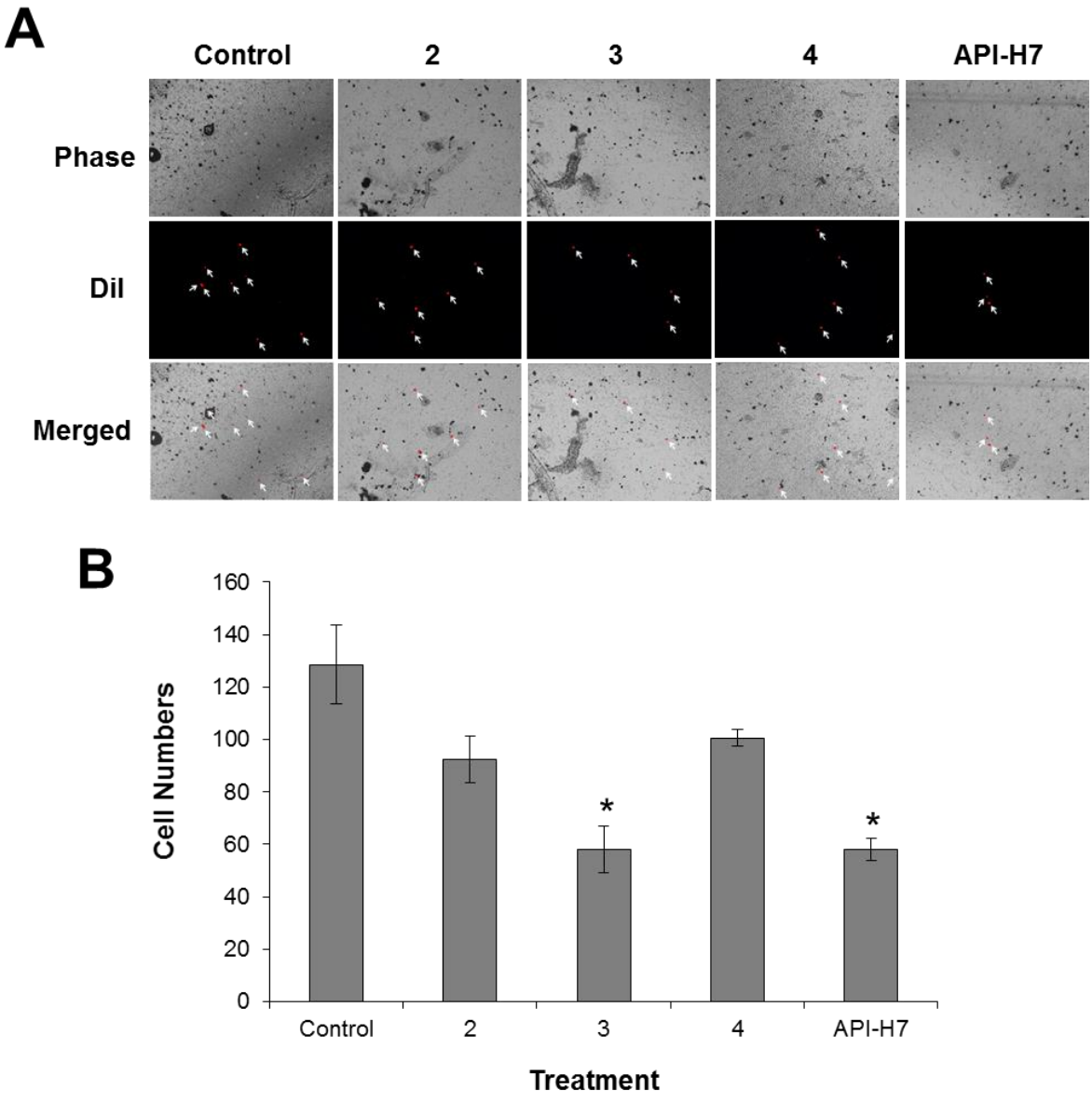

Figure S3. Dose-dependent antitumor activity of quercetin 3-*O*-glucoside (**3**) in vivo. A) Representative phase contrast, fluorescent and merged images of HCT116 cancer cells extracted from enzymatically dissociated embryos. Xenografted embryos were treated with 20 μM , 40 μM or 80 μM **3** for 4 days (7 animals/treatment). Arrows indicate extracted cancer cell. B) Dose-dependent effects of **3** on cancer cell proliferation in vivo. DMSO and 10 μM API-H7 were used as negative and positive controls, respectively. Error=SEM; *=P<0.05 compared to the negative control group.

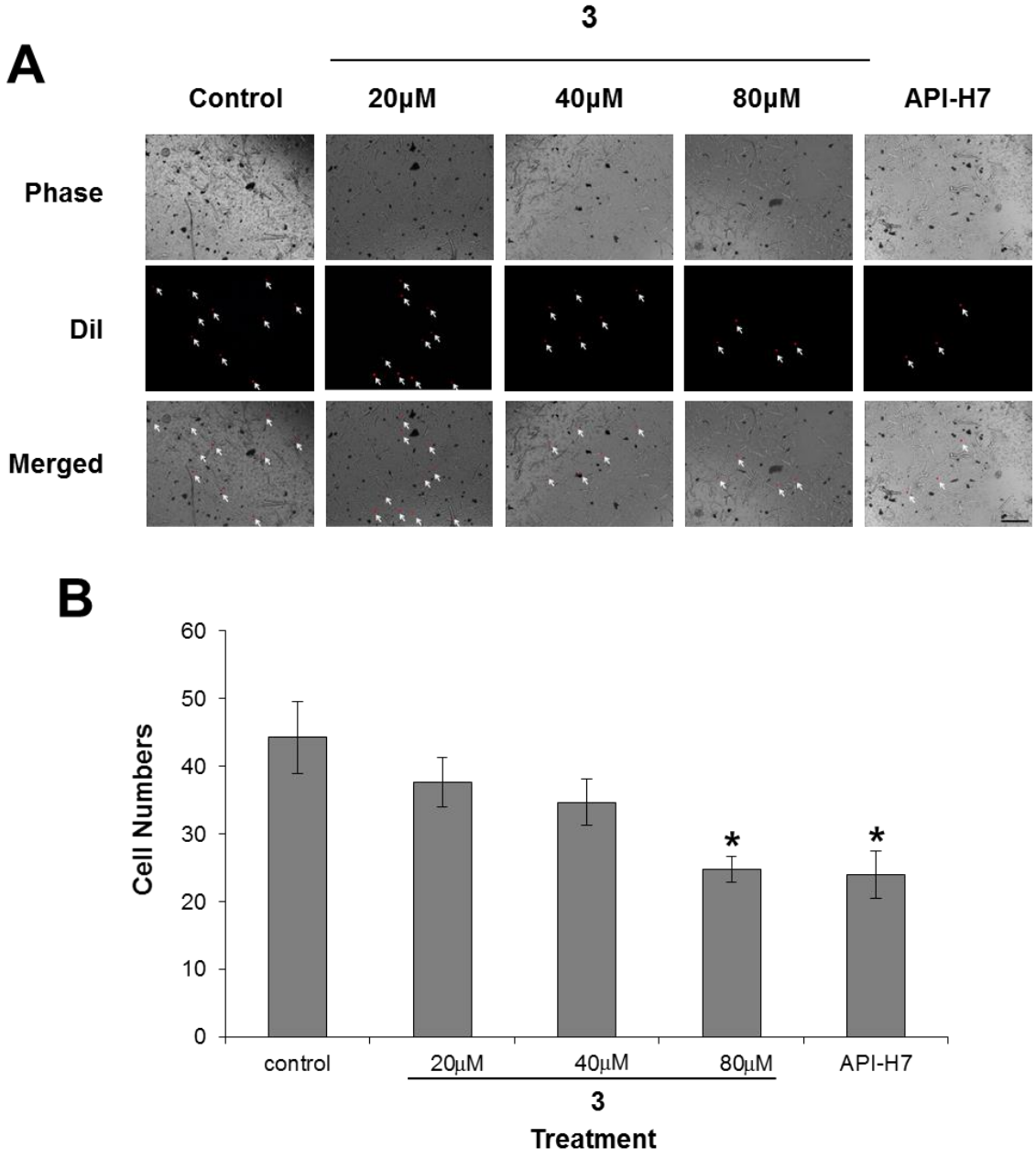

Figure S4. Detection of deglycosylation of quercetin glycoside (**2**-**4**) in vivo. 7 dpf zebrafish larvae were incubated with Res-β-Glc in the presence or absence of 80 μM **2**, **3**, **4**, and 10 μM API-H7 for 2 h (22 animals/treatment, *n*=3). The larvae were lysed for measuring fluorescent intensity. DMSO served as a negative control. Reduced fluorescence intensity showed that deglycosylation of Res-β-Glc was inhibited competitively in the presence of **3**. In contrast, **2** and **4** did not quench probe fluorescence. API-H7, which has no sugar moiety in the structure, did not affect the fluorescence derived from Res-β-Glc. Error=SEM; *=P<0.05 compared to DMSO-treated control group.