JOURNAL OF NATURAL PRODUCTS

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Antitumor Agents. 289. Design, Synthesis, and Anti-Breast Cancer Activity in Vivo of 4-Amino-2*H*-benzo[*h*]chromen-2-one and 4-Amino-7,8,9,10-tetrahydro-2*H*-benzo[*h*]chromen-2-one Analogues with Improved Water Solubility

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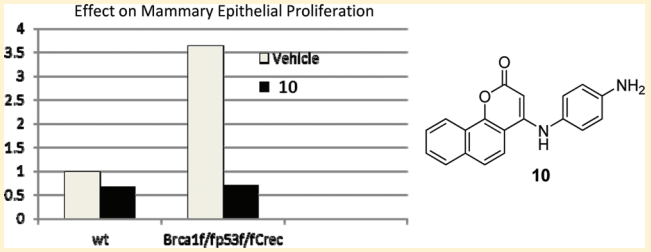
ABSTRACT: Previously, we reported that 4-amino-2*H*-benzo[*h*]chromen-2-one (ABO) and 4-amino-7,8,9,10-tetrahydro-2*H*-benzo[*h*]chromen-2-one (ATBO) analogues, which were developed from the lead natural product *neo*-tanshinlactone, are potent cytotoxic agents. In order to improve on their water solubility, the diamino analogues and related salts were designed. All synthesized compounds were assayed for cytotoxicity, and selected compounds were evaluated for in vivo anti-mammary epithelial proliferation activity in wild-type mice and mice predisposed for mammary tumors due to Brca1/p53 mutations. The new derivatives **10**, **16** (ABO), **22**, and **27** (ATBO) were the most active analogues, with IC_{50} values of 0.038−0.085 μM in the cytotoxicity assay. Analogue **10** showed around 50-fold improved water solubility compared with the prior lead ABO compound 4-[(4′-methoxyphenyl)amino]-2*H*-benzo[*h*]chromen-2-one (**3**). Compounds **3**, **4**, **10**, and **22** significantly reduced overall numbers of mammary cells, as indicated by the reduction of mammary gland branching in mutant mice. A one-week treatment with **10** resulted in 80% reduction in BrdU-positive cells in the cancer prone mammary gland. These four compounds had differential effects on cellular proliferation and apoptosis in wild-type mouse and a mouse model of human breast cancers. Compound **10** merits further development as a promising anticancer clinical trial candidate.



Water solubility of a drug and its behavior in water are critical to the bioavailability of pharmacological preparations.[1] Drugs administered orally as a solid must first dissolve in the aqueous gastric fluid and then can be absorbed and transported through the systemic circulation to their site of action.[2] Even when drugs enter the circulatory system, their water solubility not only can influence subsequent bioavailability but also may lead to side effects such as crystallization in the kidney, possibly resulting in kidney damage.[3] Therefore, water-soluble derivatives could have superior pharmacokinetic properties compared with poorly soluble ones. Due to the importance of this issue, new analogues with a reasonable degree of water solubility must be designed at an early stage of drug development. Since water solubility depends on a compound's chemical structure, the structures and incorporated functional groups may be modified to improve the water solubility.[4,5] A useful approach is to install polar functional groups, such as acidic or basic groups, which can also be converted to salts that have better water solubility.[6,7]

4-Amino-2*H*-benzo[*h*]chromen-2-one (ABO, **1**) and 4-amino-7,8,9,10-tetrahydro-2*H*-benzo[*h*]chromen-2-one (ATBO, **2**) analogues were previously reported as potent cytotoxic agents. These compounds were developed based on *neo*-tanshinlactone, a natural product isolated from the Chinese drug "Tanshen" (*Salvia miltiorrhiza* Bunge; Lamiaceae) (Figure 1).[8,9] The specific compounds **3** and **4** were extremely potent against a panel of human tumor cell lines, with nanomolar IC_{50} values. Moreover, the synthetic pathway to the analogues was quite efficient, which greatly increases their chemical availability. However, these prior inhibitory compounds have limited water solubility, which could adversely affect their in vivo efficacy and make formulation difficult. Previous studies also suggested that compounds with an amino functional group, which can impart higher polarity and be converted to a salt

Special Issue: Special Issue in Honor of Gordon M. Cragg

Received: September 28, 2011
Published: February 3, 2012

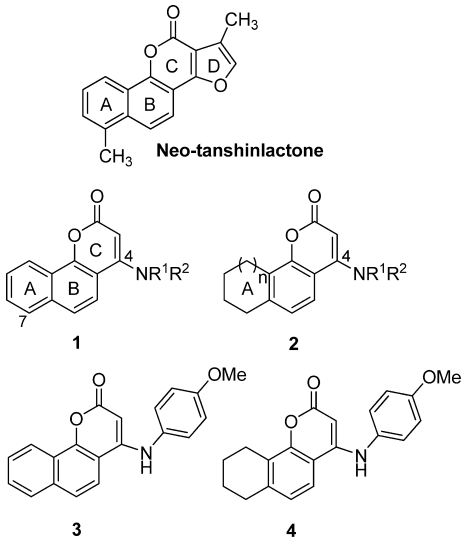


Figure 1. Structures of natural product *neo*-tanshinlactone, ABO (**1**)/ATBO (**2**) scaffolds, and lead compounds **3** and **4**.

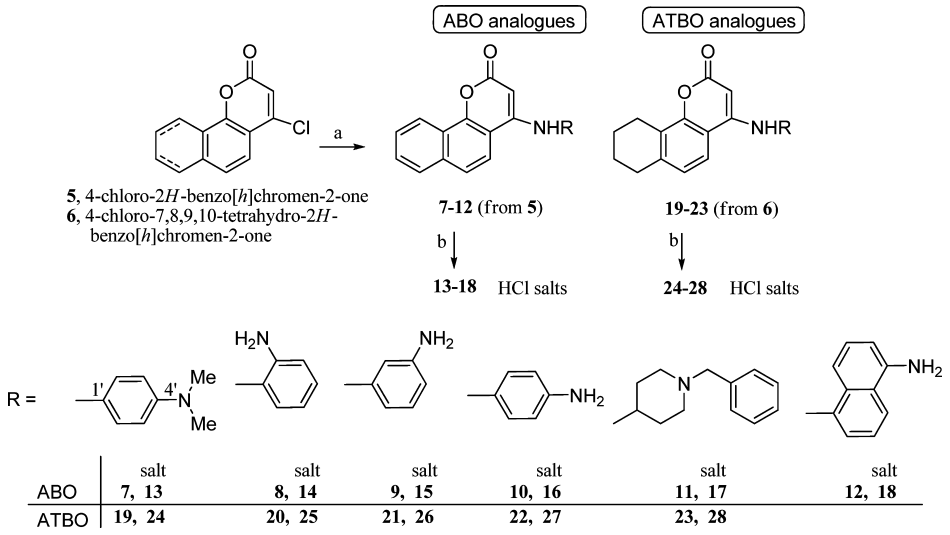
form, exhibited improved water solubility without any loss in inhibitory potency.[7] Motivated by these results, a series of diamino analogues was designed to improve water solubility and explore the structure−activity relationships (SAR). The anti-mammary epithelial proliferation and apoptosis-promoting activities of the lead compounds were examined with in vivo wild-type and Brca1/p53 mouse mammary models of human breast cancer. Individuals who inherit a mutation of either the *BRCA1* or *p53* gene have an increased risk of breast cancer. Brca1/p53 mutant mice are predisposed to mammary tumor. The mammary gland of Brca1/p53 mutant mice undergoes extensive proliferation and ductal branching.[10] Herein, the design and synthesis of new diamino analogues, the effect of amino groups on water solubility, and the antitumor activity of lead compounds both in vitro and in vivo are reported.

On the basis of previous experience, the R group in Scheme 1 can accommodate various substituents without dramatic drops in antitumor potency. Therefore, analogues **7**−**12** and **19**−**23** were designed to increase overall polarity and allow further conversion to the related salts. Aniline, piperidine, and aminonaphthylene groups were incorporated to study the effect of amino position and group size. All target compounds, **7**−**12** and **19**−**23**, as well as their salts, **13**−**18** and **24**−**28**, were synthesized from chlorides **5** and **6** according to methods reported before (Scheme 1).[8] Treatment of **5** and **6** with various amines afforded the related diamino analogues **7**−**12** and **19**−**23**, respectively, which were converted to their salt forms, **13**−**18** and **24**−**28**, respectively, with 3 N HCl in methanol.

All synthesized analogues, **7**−**28**, were tested for in vitro cytotoxic activity against a panel of human tumor cell lines according to previously published methods (Table 1).[11] Cell lines included KB (nasopharyngeal carcinoma), KB-vin (vincristine-resistant MDR KB subline), A549 (non-small-cell lung cancer), DU145 (prostate cancer cell line), and SK-BR-3 (estrogen receptor negative, HER2 overexpressing breast cancer).

Among the ABO analogues, the 4′-aniline analogue **10** was the most potent compound, with IC_{50} values of 0.038−0.055 μM. It was about 10-fold more active than **7**, suggesting that the presence of an unsubstituted primary amine is preferable to a dimethyl-substituted tertiary amine. Analogue **10** was also about 2-fold more active than **9** and significantly more active than **8**. Thus, the rank order of activity based on position of the NH_2 group was 4′- (**10**) > 3′- (**9**) ≫ 2′- (**8**) anilino. The piperidine analogue **11** showed only moderate activity, while the aminonaphthalene analogue **12** was equally potent to the 3′-anilino analogue **9**. These results indicated that the aromatic ring connected directly at the 4-amino position led to better activity. The salt forms **13**−**18** also displayed potent cytotoxic activity, comparable to that of their free bases **7**−**12**. The ATBO analogues, **19**−**23**, and the salt forms, **24**−**28**, exhibited similar SAR to the ABO analogues. With IC_{50} values of 0.053−0.074 μM, **22** and its salt form **27** were the most potent ATBO analogues and showed similar or slightly lower activity compared with the corresponding ABO analogues **10** and **16**. This finding suggested that the aromaticity of ring A was not strongly correlated with the activity.

Scheme 1[a]



[a]Reagents and conditions: (a) amine, EtOH, reflux (ethylene glycol for **11** and **12**, 160 °C); (b) 3 N HCl, MeOH.

dx.doi.org/10.1021/np2007878 | *J. Nat. Prod.* 2012, 75, 370−377

Table 1. Cytotoxicity of 3, 4, and 7−28 against a Human Tumor Cell Line Panel[a]

compd	KB	KB-vin	A549	DU145	SKBR-3
3	0.11 ± 0.01	0.13 ± 0.02	0.17 ± 0.03	0.11 ± 0.02	0.13 ± 0.01
4	0.037 ± 0.005	0.046 ± 0.002	0.049 ± 0.005	0.038 ± 0.005	0.064 ± 0.027
ABO free bases					
7	0.60 ± 0.13	0.35 ± 0.07	0.43 ± 0.10	0.36 ± 0.07	0.30 ± 0.09
8	>10	>10	>10	>10	>10
9	0.12 ± 0.009	0.16 ± 0.006	0.19 ± 0.02	0.15 ± 0.02	0.15 ± 0.01
10	0.054 ± 0.013	0.044 ± 0.004	0.055 ± 0.004	0.040 ± 0.005	0.038 ± 0.007
11	8.3 ± 2.3	7.6 ± 2.5	>10	>10	>10
12	0.16 ± 0.01	0.15 ± 0.004	0.16 ± 0.002	0.15 ± 0.02	0.20 ± 0.02
ABO HCl salts					
13	0.47 ± 0.17	0.41 ± 0.19	0.59 ± 0.06	0.30 ± 0.11	0.27 ± 0.09
14	8.2 ± 3.3	9.0 ± 2.7	9.1 ± 2.1	8.7 ± 2.3	9.5 ± 2.0
15	0.17 ± 0.03	0.15 ± 0.01	0.15 ± 0.03	0.18 ± 0.01	0.24 ± 0.06
16	0.048 ± 0.005	0.047 ± 0.002	0.054 ± 0.003	0.049 ± 0.004	0.053 ± 0.011
17	>10	9.4 ± 1.0	>10	>10	>10
18	0.21 ± 0.04	0.17 ± 0.01	0.24 ± 0.06	0.18 ± 0.01	0.24 ± 0.06
ATBO free bases					
19	0.72 ± 0.11	0.54 ± 0.04	0.54 ± 0.14	0.47 ± 0.02	0.77 ± 0.20
20	4.9 ± 0.50	5.2 ± 0.14	5.0 ± 0.35	5.0 ± 0.23	5.2 ± 0.27
21	0.44 ± 0.08	0.47 ± 0.03	0.40 ± 0.04	0.44 ± 0.05	0.48 ± 0.05
22	0.056 ± 0.004	0.053 ± 0.001	0.058 ± 0.001	0.053 ± 0.006	0.074 ± 0.016
23	6.5 ± 1.6	6.2 ± 1.4	7.0 ± 2.1	5.8 ± 1.9	7.7 ± 1.4
ATBO HCl salts					
24	0.38 ± 0.02	0.39 ± 0.03	0.44 ± 0.04	0.33 ± 0.15	0.49 ± 0.07
25	7.4 ± 0.21	7.0 ± 0.10	8.0 ± 6.4	>10	8.7 ± 1.8
26	0.48 ± 0.03	0.45 ± 0.03	0.50 ± 0.04	0.34 ± 0.10	0.56 ± 0.03
27	0.069 ± 0.011	0.065 ± 0.014	0.062 ± 0.006	0.066 ± 0.011	0.085 ± 0.023
28	9.1 ± 1.1	8.3 ± 1.1	>10	8.3 ± 1.6	9.3 ± 1.3

[a]Mean IC_{50} ± standard error (μM), from three or more independent tests. For explanation of cell lines, see text.

In order to investigate the water solubility of the new analogues, lead compound **10** was selected and its water solubility was measured using an HPLC assay,[12] with **3** and 1-naphthol as controls. As shown in Table 2, **10** (33.9 mg/L) was about 50-fold more water-soluble than **3** (0.69 mg/L). The measured water solubility of 1-naphthol was 1050 mg/L compared with 1350 mg/L reported in the literature.[13]

Table 2. Water Solubility of 3, 10, and 1-Naphthol

compd	water solubility (mg/L)
3	0.69
10	33.9
1-naphthol	1050 (1350)

Subsequently, lead compounds **3**, **4**, **10**, and **22** were selected for evaluation of in vivo antiproliferative activity. In addition to studying the effects of these compounds on wild-type mammary glands, the effects on Brca1/p53-mutated glands, which have extensive proliferation and ductal branching and are cancer-prone,[10,11] were investigated for comparison. The vehicle-treated Brca1/p53-mutated glands had significantly more branching, a phenotype similar to the untreated Brca1/p53 mutant mouse.[10] All four tested compounds resulted in considerable reduction of branching in the mutant mouse. Specifically, lead compounds **3**, **4**, **10**, and **22** reduced mammary gland branching in the Brca1/p53-mutated glands by 75%, 65%, 69%, and 70%, respectively, after 10 days of daily injection of 0.1 mg of the compound (Figure 2). These compounds also reduced branching in the wild-type mice, but to lesser degrees, ranging from 15−46%.

Interestingly, BrdU-positive populations, which are indicative of cells undergoing DNA synthesis, were significantly reduced by 55% and 81% in mutant mice treated with compounds **3** and **10**, respectively, relative to vehicle-treated mice. The effects were not pronounced in the mammary gland of wild-type mice (Figure 3). However, the BrdU-positive populations in mutant mice increased approximately 90% upon treatment with **4**, but did not change appreciably upon treatment with **22**. These latter results indicate that compound **4** could enhance cell proliferation and might not be best for the prevention or treatment of mammary tumor.

The total cell numbers are a net result of cell proliferation and apoptosis. Activated cleaved caspase-3 is required for the execution of apoptosis.[14,15] Immunohistochemical staining with antibodies recognizing cleaved caspase-3 was performed using the paraffin-embedded mammary gland.[16] Compounds **10** and **22** induced 4.8- and 4.5-fold increases, respectively, in the numbers of apoptotic cells in the Brca1/p53 mutant gland compared to vehicle-treated glands (Figure 4). In the wild-type mice, 2.1- and 4.0-fold increases in apoptosis were seen after treatment with compounds **10** and **22**, respectively. No conclusive data were obtained from **3**- and **4**-treated mice due to the high background staining (data not shown). While the dosage responses and the effects on tumors remain to be studied, the in vivo data show that both compounds **10** and **22** reduced branching and increased apoptosis (Figures 2 and 4); however, only compound **10** reduced cell proliferation (Figure 3). Compound **22** significantly increased apoptosis in the

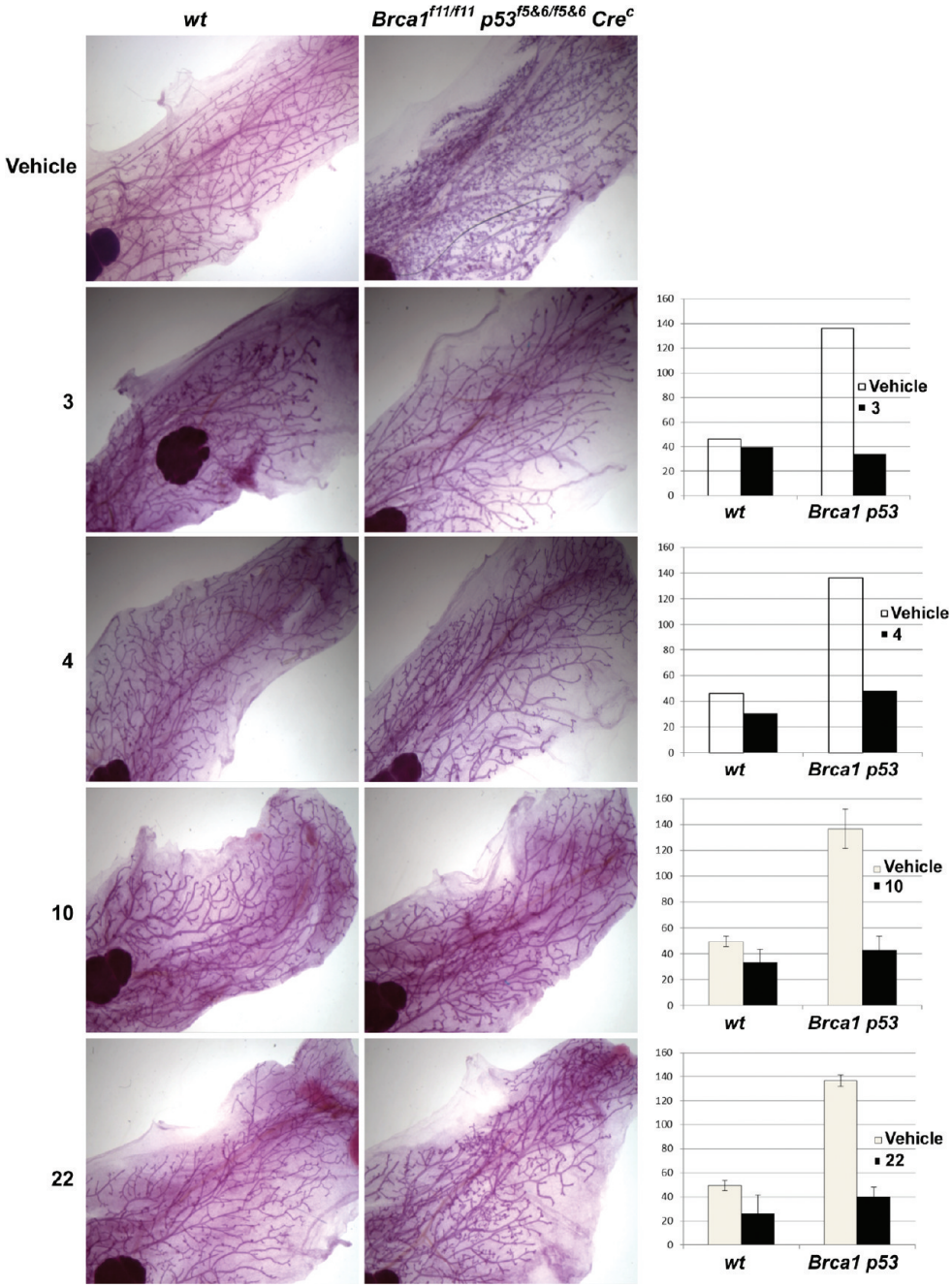


Figure 2. Mammary gland whole mounts (left) of vehicle and **3**-, **4**-, **10**-, and **22**-treated 3-month-old mice and number of branching points (right) in wild-type (*wt*) and *Brca1*$^{f11/f11}$*p53*$^{f5\&6/f5\&6}$*Cre*^{c} mammary glands after treatment with 0.1 mg of compound daily for 10 days. Compounds **3**, **4**, **10**, and **22** decreased mammary ductal branching, especially in *Brca1*$^{f11/f11}$*p53*$^{f5\&6/f5\&6}$*Cre*^{c} mice.

mammary glands of both wild-type and mutant mice. Taken together, further studies using compound **10** are warranted.

EXPERIMENTAL SECTION

General Experimental Procedures. ^{1}H NMR spectra were measured on a 300 or 400 MHz Varian Gemini 2000 spectrometer using TMS as internal standard. The solvent used was $CDCl_3$ unless indicated. Mass spectra were measured on a Shimadzu LC-MS2010 instrument. Thin-layer chromatography (TLC) and preparative TLC were performed on precoated silica gel GF plates purchased from Merck, Inc. Isco Companion systems were used for flash chromatography. Silica gel (200−400 mesh) from Aldrich, Inc., was used for column chromatography. All other chemicals were obtained from Aldrich, Inc., and Fisher, Inc. All compounds were >95% pure on the basis of HPLC conditions.

Spectroscopic and Analytical Data for New Compounds. *4-{[4′-(Dimethylamino)phenyl]amino}-2H-benzo[h]-chromen-2-one (**7**):* ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.27 (s, 1H, N*H*), 8.38 (d, 1H, J = 6.8 Hz, Ar-*H*), 8.25 (d, 1H, J = 8.8 Hz, Ar-*H*), 8.04−8.06 (m, 1H, Ar-*H*), 7.88 (d, 1H, J = 9.2 Hz, Ar-*H*), 7.71−7.74 (m, 2H, Ar-*H*), 7.20 (d, 2H, J = 9.2 Hz, Ar-*H*), 6.83 (d, 2H, J = 9.2 Hz, Ar-*H*), 5.14 (s, 1H, 3-*H*), 2.95 (s, 6H, N(C*H*$_3$)$_2$); HRMS m/z [M$^+$ + 1] calcd for $C_{21}H_{18}N_2O_2$, 331.1446, found 331.1451; HPLC (80% ACN) 99.8%; t_R 1.944 min.

*4-[(2′-Aminophenyl)amino]-2H-benzo[h]chromen-2-one (**8**):* ^{1}H NMR (400 MHz, CD_3OD) δ 8.52 (d, 1H, J = 6.8 Hz, Ar-*H*), 8.12 (d, 1H, J = 8.8 Hz, Ar-*H*), 8.00 (d, 1H, J = 8.0 Hz, Ar-*H*), 7.86 (d, 1H, J = 8.8 Hz, Ar-*H*), 7.69−7.71 (m, 2H, Ar-*H*), 7.13−7.20 (m, 2H, Ar-*H*),

dx.doi.org/10.1021/np2007878 | *J. Nat. Prod.* 2012, 75, 370−377

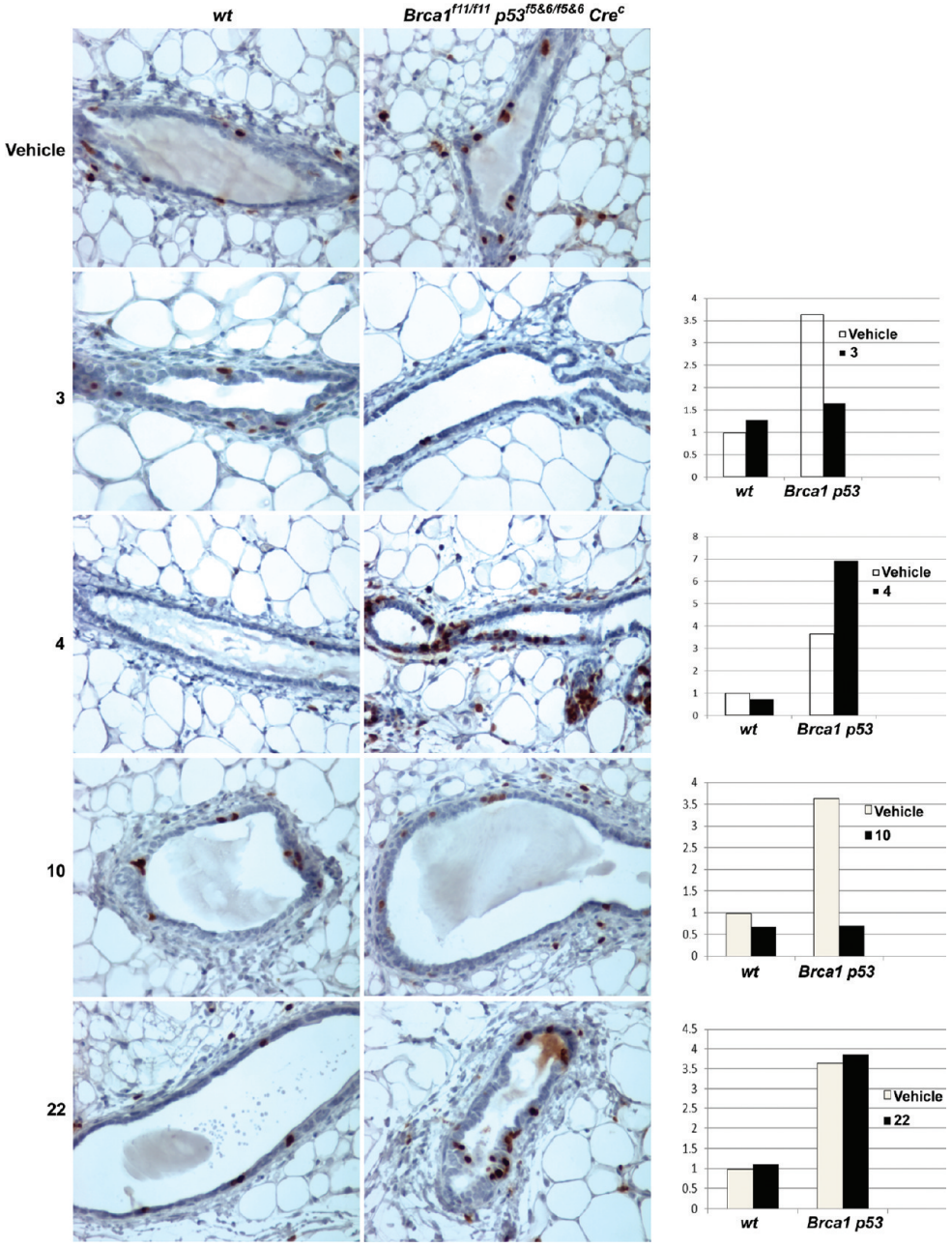


Figure 3. Effects of compounds **3**, **4**, **10**, and **22** on mammary epithelial proliferation. BrdU-containing drinking water was provided during the last three days of treatment. Cells that took up BrdU, indicative of DNA synthesis, were detected by immunostaining (left). BrdU-positive cells in 15 mammary ducts were quantified as the average numbers of BrdU-positive cells in vehicle and **3**-, **4**-, **10**-, and **22**-treated wild-type (*wt*) and *Brca1*$^{f11/f11}$*p53*$^{f5\&6/f5\&6}$*Cre*^{c} mice (right).

6.92 (d, 1H, J = 8.0 Hz, Ar-*H*), 6.78 (t, 1H, J = 7.6 Hz, Ar-*H*), 5.08 (s, 1H, 3-*H*); HRMS m/z [M$^+$ − 1] calcd for $C_{19}H_{14}N_2O_2$, 301.0977, found 301.0985; HPLC (70% ACN) 99.9%; t_R 1.817 min.

4-[(3′-Aminophenyl)amino]-2H-benzo[h]chromen-2-one (***9***): ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.24 (s, 1H, N*H*), 8.39 (d, 1H, J = 6.4 Hz, Ar-*H*), 8.25 (d, 1H, J = 8.8 Hz, Ar-*H*), 8.05 (d, 1H, J = 6.4 Hz, Ar-*H*), 7.89 (d, 1H, J = 8.8 Hz, Ar-*H*), 7.73 (s, 2H, Ar-*H*), 7.13 (t, 1H, J = 8.0 Hz, Ar-*H*), 6.60 (s, 1H, Ar-*H*), 6.51 (s, 2H, Ar-*H*), 5.43 (s, 1H, 3-*H*), 5.33 (s, 2H, NH_2); HRMS m/z [M$^+$ − 1] calcd for $C_{19}H_{14}N_2O_2$, 301.0977, found 301.0983; HPLC (70% ACN) 99.6%; t_R 1.776 min.

4-[(4′-Aminophenyl)amino]-2H-benzo[h]chromen-2-one (***10***): ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.19 (s, 1H, N*H*), 8.37 (d, 1H, J = 7.6 Hz, Ar-*H*), 8.24 (d, 1H, J = 9.2 Hz, Ar-*H*), 8.04 (d, 1H, J = 6.8 Hz, Ar-*H*), 7.87 (d, 1H, J = 8.4 Hz, Ar-*H*), 7.71−7.75 (m, 2H, Ar-*H*), 7.03 (d, 1H, J = 8.0 Hz, Ar-*H*), 6.67 (d, 1H, J = 8.0 Hz, Ar-*H*), 5.27 (s, 1H, N*H*), 5.09 (s, 1H, 3-*H*); HRMS m/z [M$^+$ − 1] calcd for $C_{19}H_{14}N_2O_2$, 301.0977, found 301.0985. HPLC (70% ACN) 98.7%; t_R 1.727 min.

4-[(1′-Benzylpiperidin-4′-yl)amino]-2H-benzo[h]chromen-2-one (***11***): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.57−8.60 m, 1H, Ar-*H*), 7.83−7.85 (m, 1H, Ar-*H*), 7.61−7.68 (m, 3H, Ar-*H*), 7.41 (d, 1H, J = 8.4 Hz, Ar-*H*), 7.26−7.34 (m, 5H, Ar-*H*), 5.43 (s, 1H, 3-*H*), 5.18 (d, 1H, J = 7.2 Hz, N*H*), 3.55 (s, 2H, 4′-NCH_2), 3.49−3.52 (m, 1H, 1′-*H*), 2.91 (d, 2H, J = 12 Hz, 3′ and 5′-*H*), 2.13−2.21 (m, 4H, 2′, 3′, 5′, and 6′-*H*), 1.66−1.72 (m, 2H, J = 12 Hz, 2′ and 6′-*H*); HRMS m/z [M$^+$ + 1] calcd for $C_{25}H_{24}N_2O_2$, 385.1916, found 385.1919; HPLC (80% ACN) 98.2%; t_R 2.167 min.

4-[(5′-Aminonaphthalen-1-yl)amino]-2H-benzo[h]chromen-2-one (***12***): ^{1}H NMR (400 MHz, CD_3OD) δ 8.53 (d, 1H, J = 8.8 Hz, Ar-*H*), 8.25 (d, 1H, J = 8.8 Hz, Ar-*H*), 8.12 (d, 1H, J = 7.2 Hz, Ar-*H*), 8.03 (d, 1H, J = 7.2 Hz, Ar-*H*), 7.91 (d, 1H, J = 8.4 Hz, Ar-*H*), 7.64−

dx.doi.org/10.1021/np2007878 | *J. Nat. Prod.* 2012, 75, 370−377

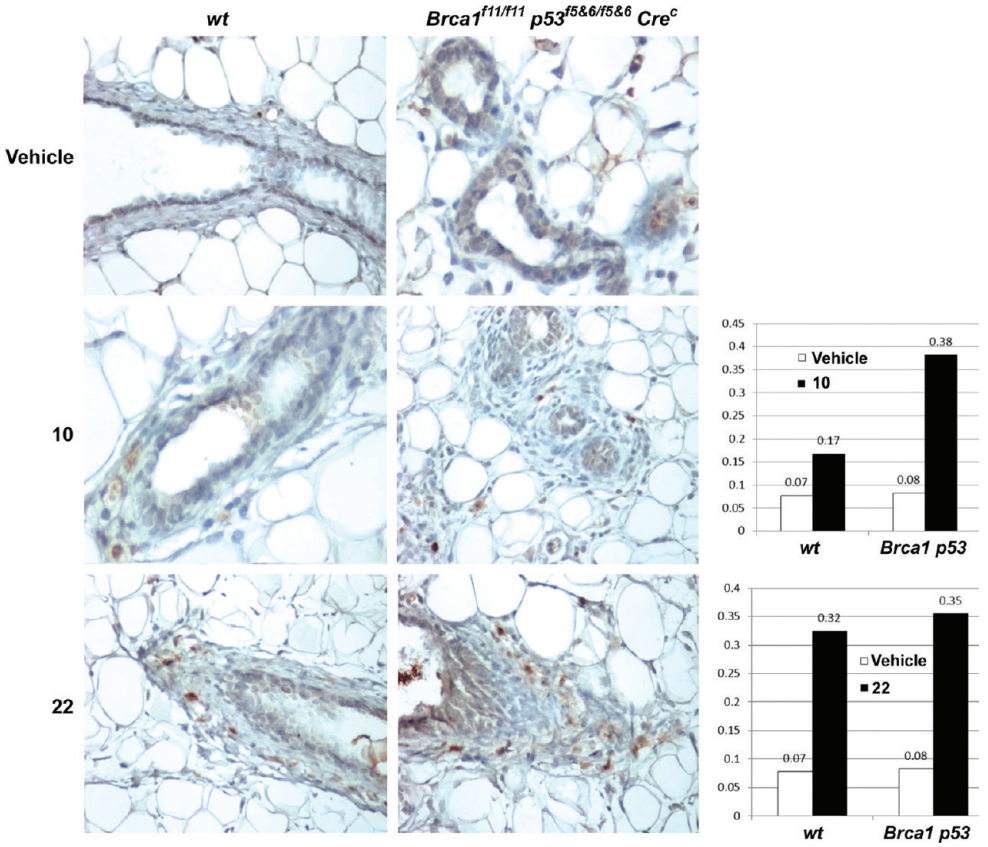


Figure 4. Immunohistochemical staining of paraffin-embedded mammary gland sections treated with compounds **10** and **22** (left) showing cytoplasmic and perinuclear localization of cleaved caspase-3. The compounds had a significant effect on mammary apoptosis as indicated by the increased percentages of activated caspase-3-positive cells/duct (right).

7.76 (m, 2H, Ar-*H*), 7.53−7.54 (m, 2H, Ar-*H*), 7.28 (s, 2H, Ar-*H*), 6.87−6.89 (m, 1H, Ar-*H*); HRMS *m*/*z* [$M^+ - 1$] calcd for $C_{23}H_{16}N_2O_2$, 351.1133, found 351.1148; HPLC (70% ACN) 99.8%; t_R 1.940 min.

*4-{[4′-(Dimethylamino)phenyl]amino}-2H-benzo[h]chromen-2-one hydrochloride (**13**):* ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.32 (s, 1H, N*H*, D_2O exchanged), 8.38 (d, 1H, *J* = 8.0 Hz, Ar-*H*), 8.26 (d, 1H, *J* = 9.2 Hz, Ar-*H*), 7.91−8.06 (m, 1H, Ar-*H*), 7.87 (d, 1H, *J* = 8.8 Hz, Ar-*H*), 7.69−7.76 (m, 2H, Ar-*H*), 7.27 (d, 2H, *J* = 8.0 Hz, Ar-*H*), 7.00 (s, 2H, Ar-*H*), 5.20 (s, 1H, 3-*H*, D_2O exchanged), 2.99 [s, 6H, $N(CH_3)_2$].

*4-[(2′-Aminophenyl)amino]-2H-benzo[h]chromen-2-one hydrochloride (**14**):* ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.15 (s, 1H, N*H*, D_2O exchanged), 8.37−8.39 (m, 1H, Ar-*H*), 8.29 (d, 1H, *J* = 9.2 Hz, Ar-*H*), 8.05−8.07 (m, 1H, Ar-*H*), 7.89 (d, 1H, *J* = 8.8 Hz, Ar-*H*), 7.70−7.76 (m, 2H, Ar-*H*), 7.15−7.20 (m, 2H, Ar-*H*), 6.96 (d, 1H, *J* = 7.6 Hz, Ar-*H*), 6.80 (t, 1H, *J* = 7.2 Hz, Ar-*H*), 4.80 (s, 1H, 3-*H*, D_2O exchanged).

*4-[(3′-Aminophenyl)amino]-2H-benzo[h]chromen-2-one hydrochloride (**15**):* ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.36 (s, 1H, N*H*), 8.38−8.40 (m, 1H, Ar-*H*), 8.27 (d, 1H, *J* = 8.8 Hz, Ar-*H*), 8.05−8.08 (m, 1H, Ar-*H*), 7.90 (d, 1H, *J* = 9.2 Hz, Ar-*H*), 7.70−7.77 (m, 2H, Ar-*H*), 7.26 (t, 1H, *J* = 8.0 Hz, Ar-*H*), 7.70−7.27 (m, 2H, Ar-*H*), 6.74−6.85 (m, 3H, Ar and NH_2), 6.51 (s, 1H, Ar-*H*), 5.49 (s, 1H, 3-*H*).

*4-[(4′-Aminophenyl)amino]-2H-benzo[h]chromen-2-one hydrochloride (**16**):* ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.43 (s, 1H, N*H*, D_2O exchanged), 8.38(d, 1H, *J* = 8.0 Hz, Ar-*H*), 8.26 (d, 1H, *J* = 8.8 Hz, Ar-*H*), 8.06−8.08 (m, 1H, Ar-*H*), 7.82 (d, 1H, *J* = 8.8 Hz, Ar-*H*), 7.71−7.76(m, 2H, Ar-*H*), 7.38 (d, 1H, *J* = 8.4 Hz, Ar-*H*), 7.21 (d, 1H, *J* = 8.0 Hz, Ar-*H*), 6.88 (s, 1H, N*H*, D_2O exchanged), 5.33 (s, 1H, 3-*H*, D_2O exchanged).

*4-[(1′-Benzylpiperidin-4′-yl)amino]-2H-benzo[h]chromen-2-one hydrochloride (**17**):* ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.22 (s, 1H, N*H*), 8.35−8.37 (m, 1H, Ar-*H*) 8.20 (d, 1H, *J* = 9.2 Hz, Ar-*H*), 8.01−8.04 (m, 1H, Ar-*H*), 7.83 (d, 1H, *J* = 9.2 Hz, Ar-*H*), 7.67−7.74 (m, 2H, Ar-*H*), 7.49−7.61 (m, 5H, Ar-*H*), 5.49 (s, 1H, 3-*H*), 4.31 (d, 2H, *J* = 4.8 Hz, 4′-NCH_2), 3.79−3.81 (m, 1H, 1′-*H*), 3.46 (d, 2H, *J* = 11.6 Hz, 3′ and 5′-*H*), 3.10 (dd, 2H, *J* = 10.4, 23.2 Hz, 3′ and 5′-*H*), 2.18 (d, 2H, *J* = 12.8 Hz, 2′ and 6′-*H*), 1.97 (dd, 2H, *J* = 12.4, 24.8 Hz, 2′ and 6′-*H*).

*4-[(5′-Aminonaphthalen-1′-yl)amino]-2H-benzo[h]chromen-2-one hydrochloride (**18**):* ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.72 (s, 1H, N*H*, D_2O exchanged), 8.38−8.43 (m, 2H, Ar-*H*), 8.16−8.18 (m, 1H, Ar-*H*), 8.10 (d, 1H, *J* = 8.0 Hz, Ar-*H*), 7.96 (d, 1H, *J* = 8.8 Hz, Ar-*H*), 7.12−7.77 (m, 2H, Ar-*H*), 7.56−7.58 (m, 2H, Ar-*H*), 7.27−7.32 (m, 2H, Ar-*H*), 6.96 (s, 1H, Ar-*H*), 4.62 (s, 1H, 3-*H*, D_2O exchanged).

*4-{[4′-(Dimethylamino)phenyl]amino}-7,8,9,10-tetrahydro-2H-benzo[h]chromen-2-one (**19**):* ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.02 (s, 1H, N*H*), 7.94 (d, 1H, *J* = 8.4 Hz, Ar-*H*), 7.14 (d, 2H, *J* = 8.8 Hz, Ar-*H*), 7.09 (d, 1H, *J* = 8.4 Hz, Ar-*H*), 6.80 (d, 2H, *J* = 9.2 Hz, Ar-*H*), 5.01 (s, 1H, 3-*H*), 2.94 (s, 6H, $N(CH_3)_2$), 2.82 (t, 2H, *J* = 5.6 Hz, 10-*H*), 2.76 (t, 2H, *J* = 6.0 Hz, 7-*H*), 1.75−1.81 (m, 4H, 8 and 9-*H*); HRMS *m*/*z* [$M^+ + 1$] calcd for $C_{21}H_{22}N_2O_2$, 335.1760, found 335.1762; HPLC (70% ACN) 97.6%; t_R 2.656 min.

*4-[(2′-Aminophenyl)amino]-7,8,9,10-tetrahydro-2H-benzo[h]-chromen-2-one (**20**):* ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.81 (s, 1H, N*H*). 7.95 (d, 1H, *J* = 8.4 Hz, Ar-*H*), 7.06−7.10 (m, 2H, Ar-*H*), 7.00 (dd, 1H, *J* = 8.0, 1.2 Hz, Ar-*H*), 6.82 (dd, 1H, *J* = 8.0, 1.2 Hz, Ar-*H*), 6.61−6.65 (m, 1H, Ar-*H*), 5.02 (s, 2H, NH_2), 4.62 (s, 1H, 3-*H*), 2.82 (t, 2H, *J* = 6.0 Hz, 10-*H*), 2.76 (t, 2H, *J* = 6.0 Hz, 7-*H*), 1.74−1.81 (m, 4H, 8 and 9-*H*); HRMS *m*/*z* [$M^+ - 1$] calcd for $C_{19}H_{18}N_2O_2$, 305.1290, found 305.1297; HPLC (85% ACN) 99.9%; t_R 1.690 min.

*4-[(3′-Aminophenyl)amino]-7,8,9,10-tetrahydro-2H-benzo[h]-chromen-2-one (**21**):* ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.99 (s, 1H, N*H*), 7.95 (d, 1H, *J* = 8.4 Hz, Ar-*H*), 7.06−7.10 (m, 2H, Ar-*H*), 6.53−6.55 (m, 1H, Ar-*H*), 6.44−6.48 (m, 2H, Ar-*H*), 5.31 (s, 1H, 3-*H*), 5.28

dx.doi.org/10.1021/np2007878 | *J. Nat. Prod.* 2012, 75, 370−377

(s, 2H, NH_2), 2.82 (t, 2H, J = 6.0 Hz, 10-*H*), 2.76 (t, 2H, J = 6.0 Hz, 7-*H*), 1.74−1.81 (m, 4H, 8 and 9-*H*); HRMS m/z [$M^+ - 1$] calcd for $C_{19}H_{18}N_2O_2$, 305.1290, found 305.1298; HPLC (70% ACN) 99.2%; t_R 1.946 min.

4-[(4′-Aminophenyl)amino]-7,8,9,10-tetrahydro-2H-benzo[h]-chromen-2-one (**22**): ^{1}H NMR (400 MHz, CD_3OD) δ 7.78 (d, 1H, J = 8.0 Hz, Ar-*H*), 7.10 (d, 1H, J = 8.4 Hz, Ar-*H*), 7.04−7.06 (m, 2H, Ar-*H*), 6.79−6.82 (m, 2H, Ar-*H*), 5.23 (s, 1H, 3-*H*), 2.88 (t, 4H, J = 6.0 Hz, 7 and 10-*H*), 1.85−1.87 (m, 4H, 8 and 9-*H*); HRMS m/z [$M^+ - 1$] calcd for $C_{19}H_{18}N_2O_2$, 305.1290, found 305.1298; HPLC (70% ACN) 99.9%; t_R 1.904 min.

4-[(1′-Benzylpiperidin-4-yl)amino]-7,8,9,10-tetrahydro-2H-benzo[h]chromen-2-one (**23**): ^{1}H NMR (400 MHz, CD_3OD) δ 7.71 (d, 1H, J = 8.4 Hz, Ar-*H*), 7.27−7.35 (m, 5H, Ar-*H*), 7.04 (d, 1H, J = 8.4 Hz, Ar-*H*), 5.27 (s, 1H, 3-*H*), 3.57 (s, 2H, 4′-NCH_2), 3.52−3.55 (m, 1H, 1′-*H*), 2.98 (d, 2H, J = 12 Hz, 3′ and 5′-*H*), 2.85 (dd, 4H, J = 11.2, 5.2 Hz, 7 and 10-*H*, 2′ and 6′-*H*), 2.19−2.25 (m, 2H, 2′ and 6′-*H*), 2.03 (d, 2H, J = 12.8 Hz, 3′ and 5′-*H*), 1.72−1.87 (m, 6H, 8, 9, 2′ and 6′-*H*); HRMS m/z [$M^+ - 1$] calcd for $C_{25}H_{28}N_2O_2$, 387.2072, found 387.2097; HPLC (60% ACN) 99.2%; t_R 4.439 min.

4-{[4′-(Dimethylamino)phenyl]amino}-7,8,9,10-tetrahydro-2H-benzo[h]chromen-2-one hydrochloride (**24**): ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.09 (s, 1H, N*H*, D_2O exchanged), 7.94 (d, 1H, J = 8.4 Hz, Ar-*H*), 7.22 (d, 2H, J = 6.8 Hz, Ar-*H*), 7.09 (d, 3H, J = 8.4 Hz, Ar-*H*), 7.01 (s, 2H, NH_2, D_2O exchanged), 5.08 (s, 1H, 3-*H*, D_2O exchanged), 2.99 [s, 6H, $N(CH_3)_2$], 2.82 (t, 2H, J = 5.6 Hz, 10-*H*), 2.76 (t, 2H, J = 6.0 Hz, 7-*H*), 1.75−1.81 (m, 4H, 8 and 9-*H*).

4-[(2′-Aminophenyl)amino]-7,8,9,10-tetrahydro-2H-benzo[h]-chromen-2-one hydrochloride (**25**): ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.92 (s, 1H, N*H*, D_2O exchanged), 7.99 (d, 1H, J = 8.4 Hz, Ar-*H*), 7.09−7.19 (m, 3H, Ar-*H*), 6.98 (t, 1H, J = 8.0 Hz, Ar-*H*), 6.82 (t, 1H, J = 8.0 Hz, Ar-*H*), 4.68 (s, 1H, 3-*H*, D_2O exchanged), 2.83 (t, 2H, J = 6.0 Hz, 10-*H*), 2.77 (t, 2H, J = 6.0 Hz, 7-*H*), 1.75−1.81 (m, 4H, 8 and 9-*H*).

4-[(3′-Aminophenyl)amino]-7,8,9,10-tetrahydro-2H-benzo[h]-chromen-2-one hydrochloride (**26**): ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.23 (s, 1H, N*H*, D_2O exchanged), 7.97 (d, 1H, J = 8.4 Hz, Ar-*H*), 7.38 (t, 1H, J = 8.0 Hz, Ar-*H*), 7.12 (d, 1H, J = 8.4 Hz, Ar-*H*), 7.05 (s, 2H, Ar-*H*), 5.42 (s, 1H, 3-*H*, D_2O exchanged), 2.83 (t, 2H, J = 6.0 Hz, 10-*H*), 2.77 (t, 2H, J = 6.0 Hz, 7-*H*), 1.74−1.81 (m, 4H, 8 and 9-*H*).

4-[(4′-Aminophenyl)amino]-7,8,9,10-tetrahydro-2H-benzo[h]-chromen-2-one hydrochloride (**27**): ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.23 (s, 1H, N*H*, D_2O exchanged), 7.96 (d, 1H, J = 8.4 Hz, Ar-*H*), 7.37 (d, 2H, J = 8.4 Hz, Ar-*H*), 7.27 (d, 2H, J = 8.4 Hz, Ar-*H*), 7.12 (d, 1H, J = 8.4 Hz, Ar-*H*), 6.93 (s, 1H, NH_2), 5.25 (s, 1H, 3-*H*, D_2O exchanged), 2.83 (t, 2H, J = 6.0 Hz, 10-*H*), 2.77 (t, 2H, J = 6.0 Hz, 7-*H*), 1.74−1.81 (m, 4H, 8 and 9-*H*).

4-[(1′-Benzylpiperidin-4-yl)amino]-7,8,9,10-tetrahydro-2H-benzo[h]chromen-2-one hydrochloride (**28**): ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.35 (s, 1H, N*H*), 7.88 (d, 1H, J = 8.4 Hz, Ar-*H*), 7.58−7.61 (m, 2H, Ar-*H*), 7.48−7.50 (m, 3H, Ar-*H*), 7.31 (d, 1H, J = 7.6 Hz, N*H*), 7.02 (d, 1H, J = 8.4 Hz, Ar-*H*), 5.31 (s, 1H, 3-*H*), 4.28 (d, 2H, J = 5.2 Hz, 4′-NCH_2), 3.70−3.72 (m, 1H, 1′-*H*), 3.26−3.77 (m, 2H, 3′ and 5′-*H*), 3.07 (dd, 2H, J = 10.4, 23.2 Hz, 3′ and 5′-*H*), 2.79 (t, 2H, J = 6.0 Hz, 10-*H*), 2.73 (t, 2H, J = 6.0 Hz, 7-*H*), 2.12 (d, 2H, J = 14.0 Hz, 2′ and 6′-*H*), 1.93 (dd, 2H, J = 11.2, 23.6 Hz, 2′ and 6′-*H*), 1.73−1.78 (m, 4H, 8 and 9-*H*).

Brca1$^{fp/fp}$p53$^{fp/fp}$Cre Mutant Mice. Generation of *Brca1$^{fp/fp}$p53$^{fp/fp}$Cre* and *p53$^{fp/fp}$Cre* mice has been described previously.[10,17] The mice were in a C57BL/6 and 129/Sv, mixed background. All animal experiments were performed in accordance with guidelines of Federal and Institutional Animal Care and Use Committee at the University of California, Irvine.

Treatment with 3, 4, 10, and 22. Three-month-old mice were treated with 0.1 mg of **3** or **4**, **10**, **22**, or vehicle daily for 10 days. The concentration of stock solution of **3** or **4**, **10**, and **22** was 1 mg/mL in dimethylsulfoxide. A mixture of 10 μL of stock solution, 30 μL of 40% polyethylene glycol, and 60 μL of 0.9% NaCl solution was prepared at the time of treatment. Vehicle includes all solution except **3**, **4**, **10**, or **22**. Vehicle or compound was administered ip every day for 11 days.

Histology and Immunohistochemistry. The fourth pair glands were dissected and spread on a glass slide. After fixation with Carnoy's fixative for 3 h, the tissues were hydrated and stained in Carmine alum overnight as described (http://mammary.nih.gov/tools/histological/Histology/index.html#a1). Branching points in three random areas totaling approximately 2 mm^2 were counted. For histological section, tissues were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) at 4 °C overnight followed by paraffin embedding. Paraffin sections were stained with hematoxylin and eosin and examined by light microscopy. Immunostaining was performed following the protocol described in the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). To retrieve the antigen, slides were heated for 20 min in 10 mM citrate buffer, pH 6.0, in a microwave oven. BrdU monoclonal antibody (GeneTex Inc., Irvine, CA, USA) at 1:1000 dilution and cyclin D1 polyclonal rat antibody (NeoMarkers/Thermo Fisher Scientific, Fremont, CA, USA) at 1:500 dilution, respectively, were used for immunostaining.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors are thankful to Dr. X. S. Wang and H. Tang of the University of North Carolina at Chapel Hill for valuable discussions. This work was supported by NIH grant CA-17625-32 from the National Cancer Institute, awarded to K.H.L. This study was also supported in part by the Taiwan Department of Health, China Medical University Hospital Cancer Research Center of Excellence (DOH100-TD-C-111-005).

DEDICATION

Dedicated to Dr. Gordon M. Cragg, formerly Chief, Natural Products Branch, National Cancer Institute, for his pioneering work on the development of natural product anticancer agents.

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