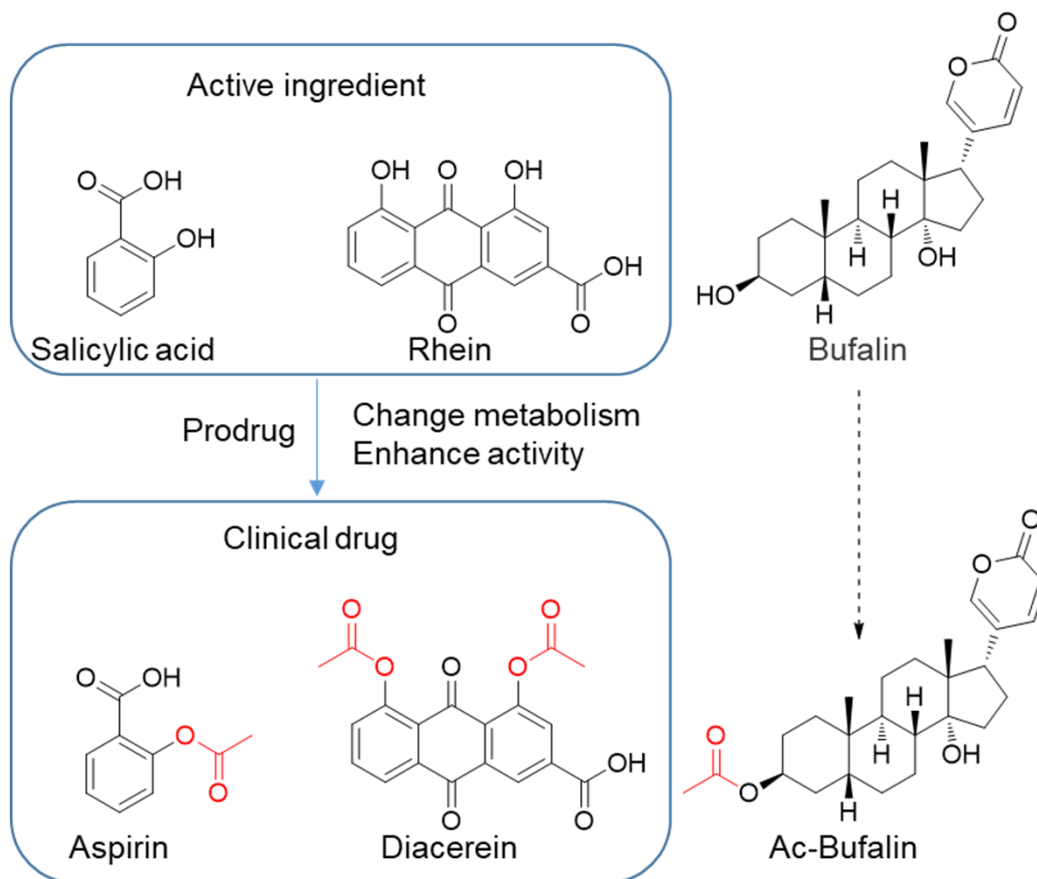


Figure S1

A



B



Figure S2

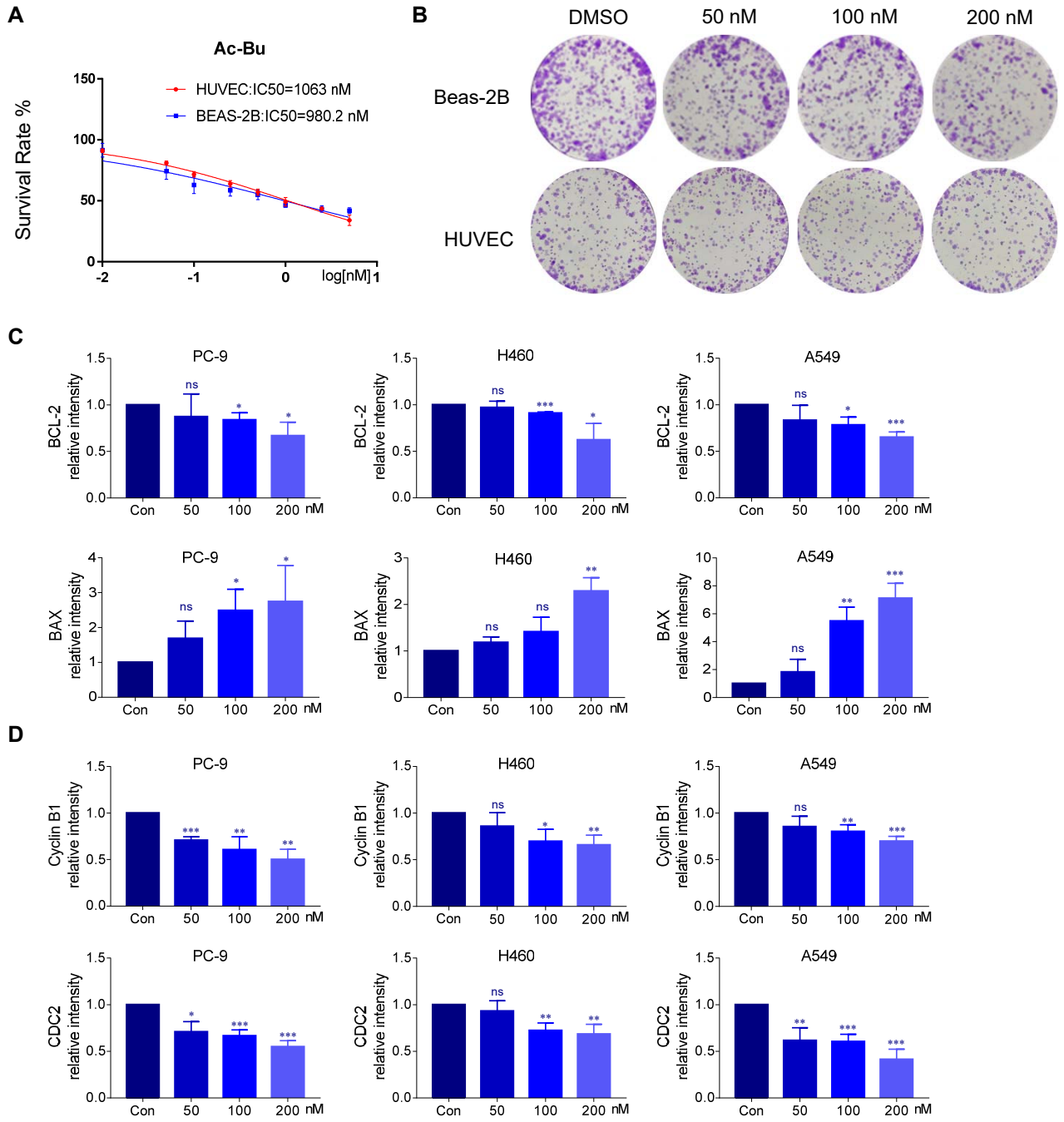
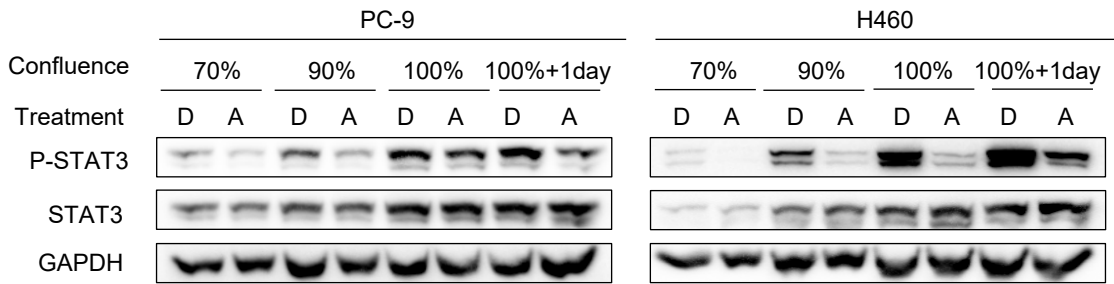
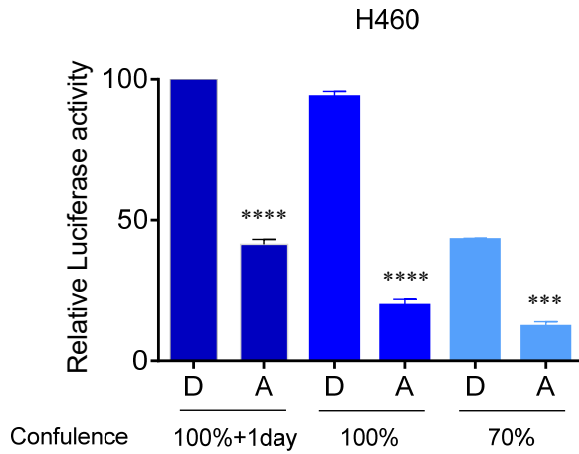


Figure S3

A



B



C

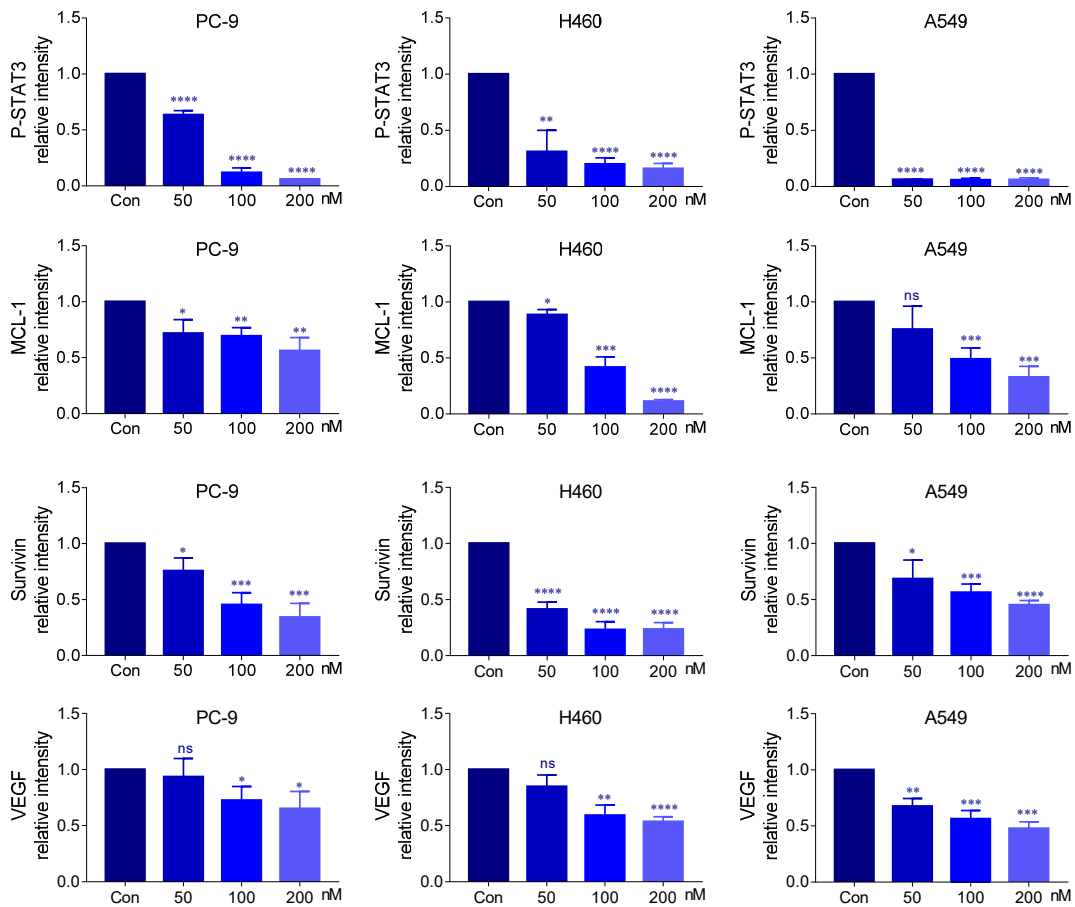
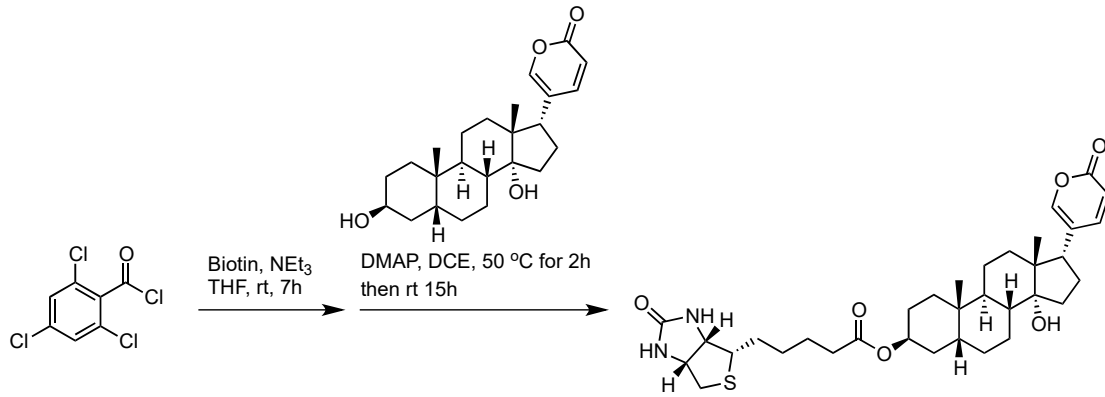
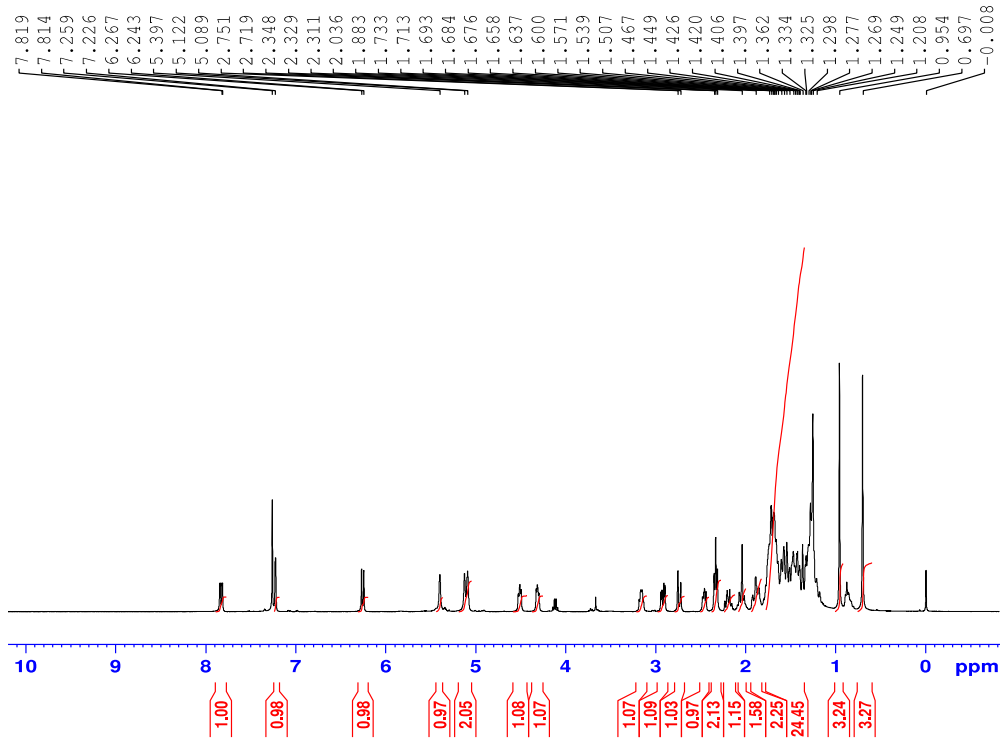


Figure S4

A



B



C

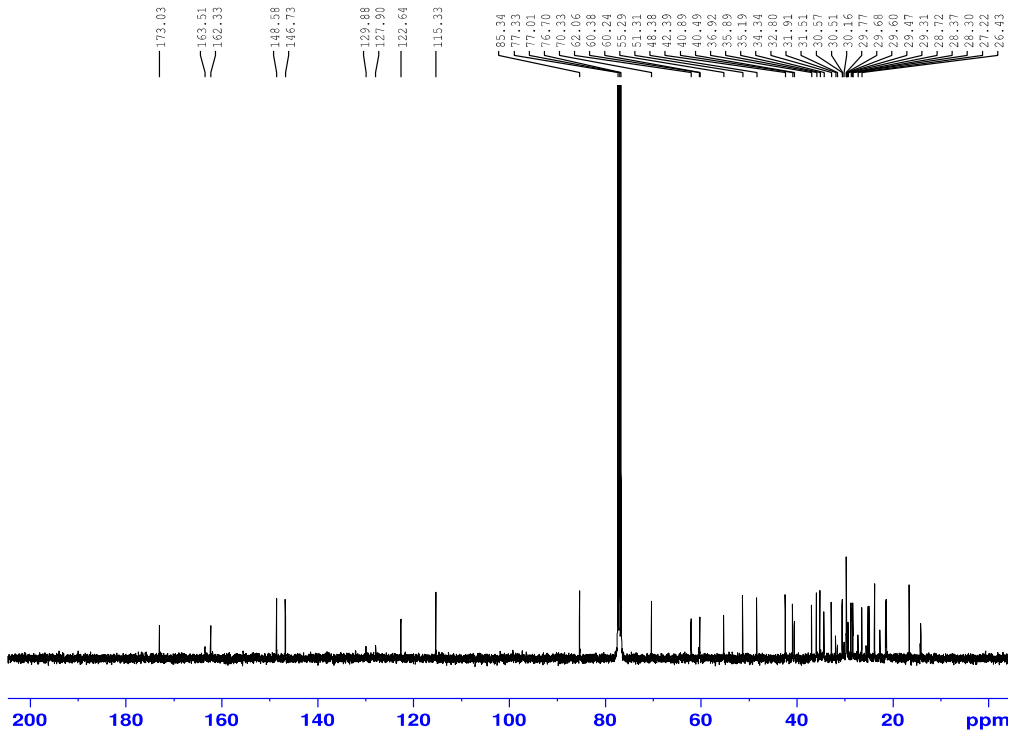


Figure S5

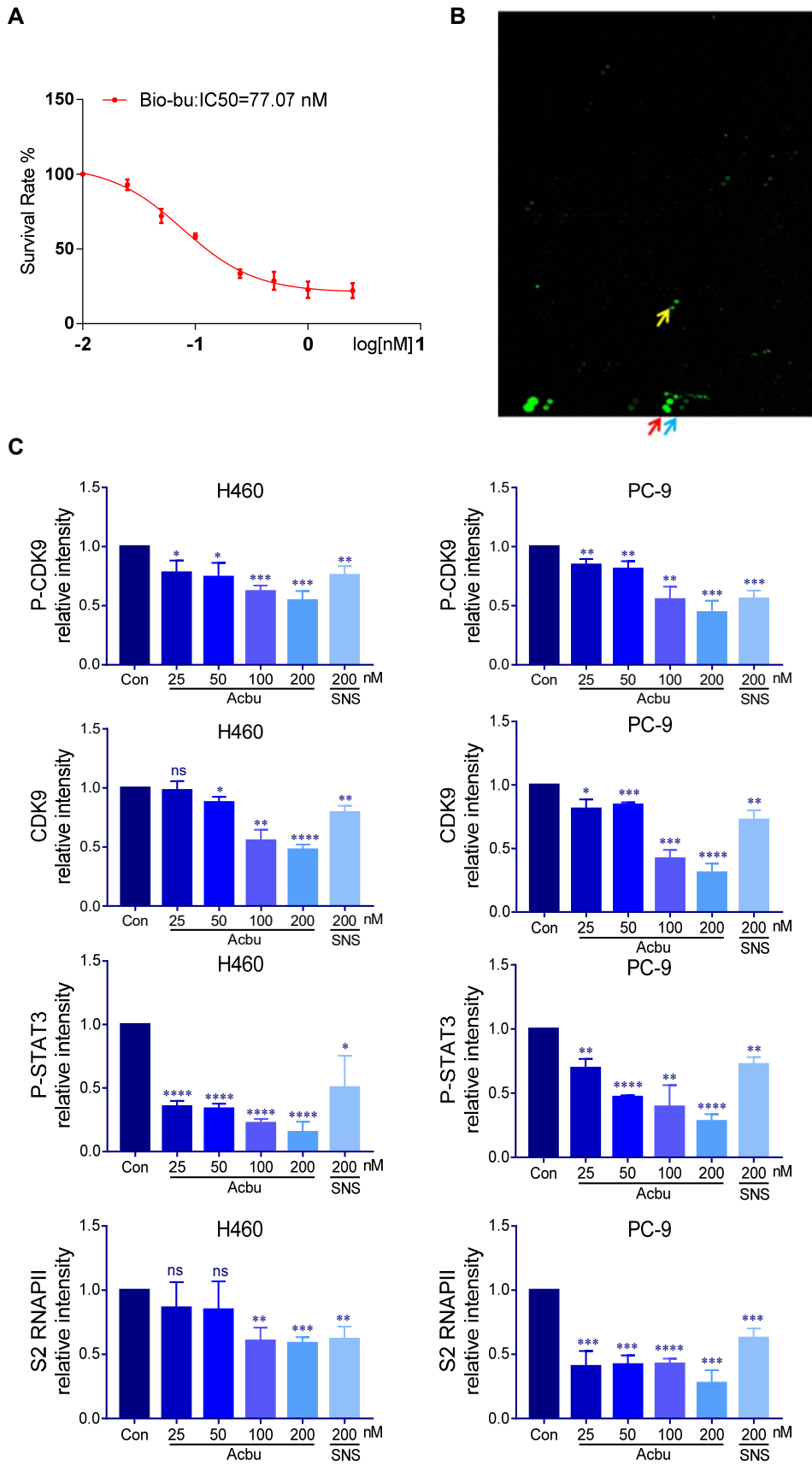


Figure S6

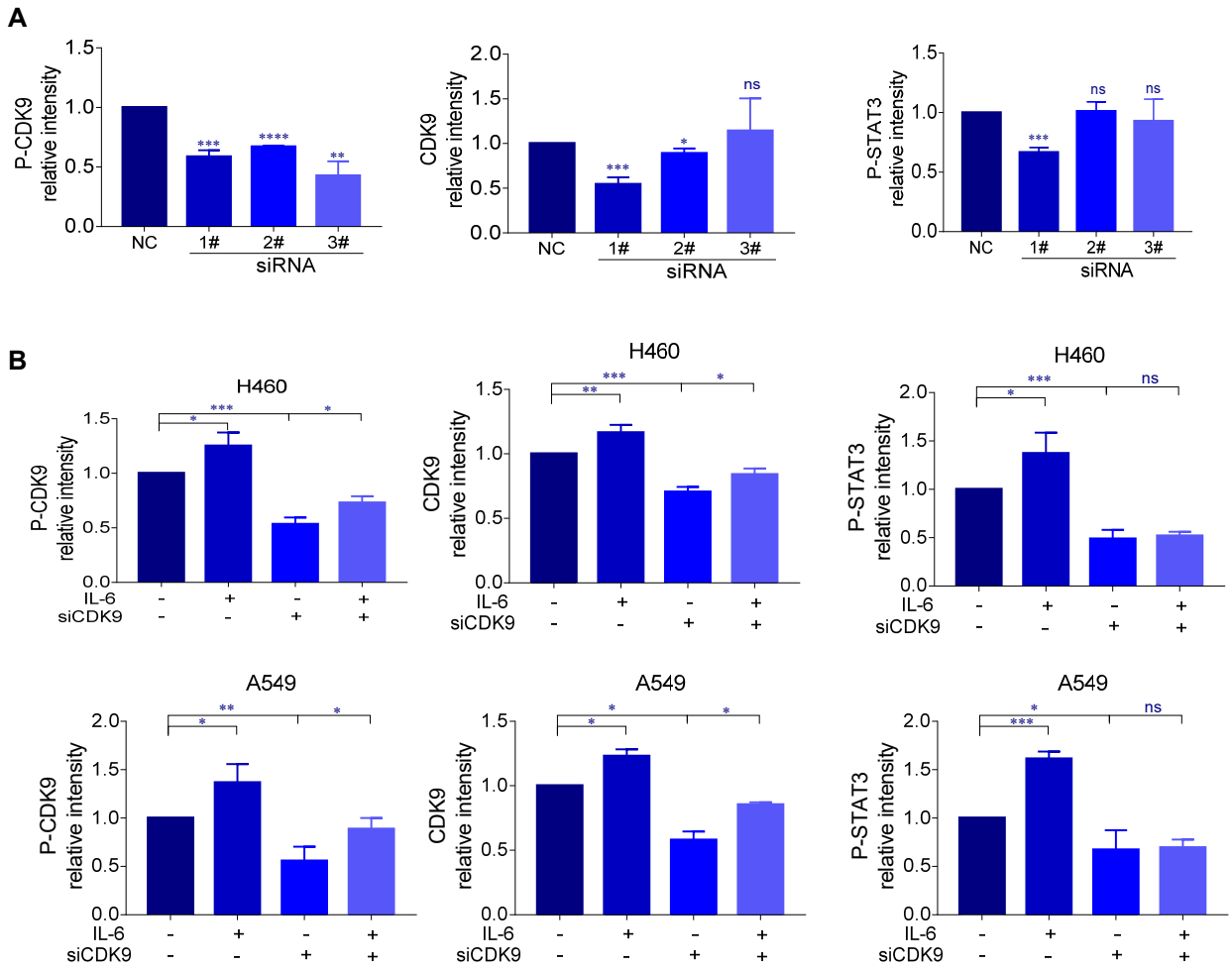
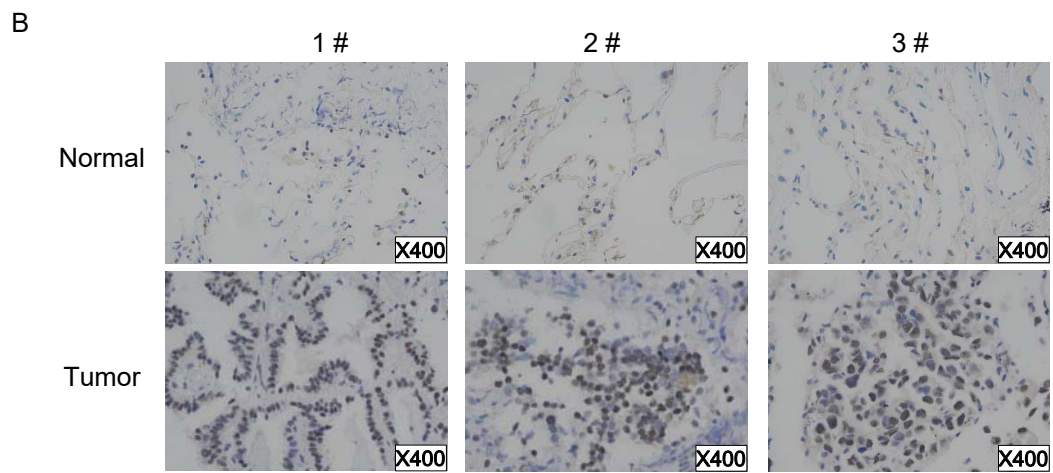
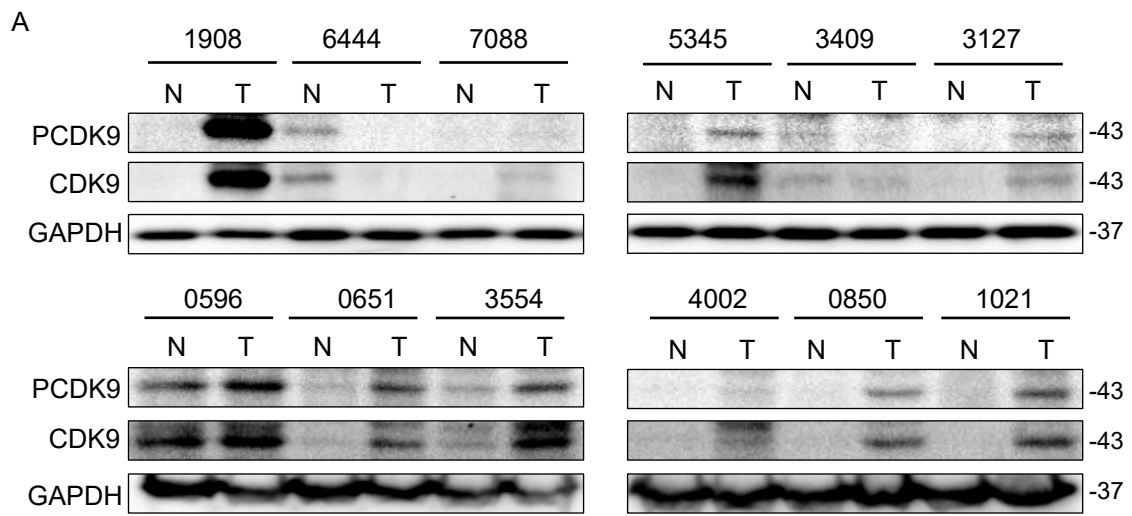


Figure S7



1 **Fig. S1 Designed and synthesized acetyl-bufalin**

2 **A.** Designed and synthesized acetyl-bufalin (Ac-bufalin) base on aspirin (acetylsalicylic acid) and
3 diacerein (diacetoxy-rhein). **B.** Chemistry experimental section of Ac-bufalin: To a mixture of
4 bufalin (15 mg, 0.0388 mmol) and NEt₃ (0.39 mmol) in DCE (dry, 1 mL) was added AcCl (0.39
5 mmol), the reaction mixture was stirred at rt for 19 h. The mixture was then purified by a silica gel
6 flash chromatography (DCM/MeOH = 50:1 to 20:1) to afford target compound (17 mg, 100% yield)
7 as a pale yellow solid. ¹H NMR (400 MHz, CDCl₃) δ 7.82 (dd, *J* = 9.6, 4.0 Hz, 1H), 7.22 (s, 1H),
8 6.26 (d, *J* = 9.6 Hz, 1H), 5.07 (s, 1H), 2.49-2.40 (m, 1H), 2.26-2.15 (m, 1H), 2.09-1.99 (m, 4H),
9 1.94-1.81 (m, 2H), 1.80-1.35 (m, 18H), 1.27 (s, 3H), 0.69 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ
10 170.7, 162.3, 148.6, 146.7, 122.6, 115.3, 85.4, 70.4, 51.3, 48.4, 42.4, 40.9, 36.8, 35.9, 35.2, 32.8,
11 30.5, 30.5, 28.7, 26.4, 25.1, 23.7, 21.5, 21.4, 21.3, 16.5; ESI-HRMS: calcd. for C₂₆H₃₇O₅⁺ (M+H)⁺
12 429.2636, found 429.2637.

13

14 **Fig. S2 Antitumor activity of acetyl-bufalin *in vitro***

15 **A.** HUVEC and BEAS-2B cells were incubated with increasing doses of acetyl-bufalin for 48 h.
16 Cell viability was determined by the MTT assay. **B.** HUVEC and BEAS-2B cells were incubated
17 with acetyl-bufalin for 24 h and allowed to form colonies for one week. Colonies were then fixed,
18 stained with crystal violet and photographed. **C.** Cells were treated with acetyl-bufalin at different
19 concentrations as indicated for 24 h, the cell lysates were processed for Western blot analysis for
20 protein expression of BCL-2 and BAX, and the relative intensity was calculated. **D.** Western blot
21 analysis was used to detect the levels of cyclin B1 and CDC2 after acetyl-bufalin treatment and the
22 relative intensity was calculated. *P<0.05, **P<0.01, ***P<0.001.

23

24 **Fig. S3 Acetyl-bufalin inhibited the STAT3 signaling pathway in human NSCLC cells**

25 **A.** PC-9 and H460 cells were grown to 70, 90 or 100% confluence and up to 1 day postconfluence
26 as indicated, in the presence of 50 nM acetyl-bufalin or the DMSO diluent. Cell lysates were used
27 to detect P-STAT3, STAT3 and GAPDH as a control. **B.** H460 cells were grown to 70, or 100%
28 confluence and up to 1 day postconfluence, then transfected with luciferase reporter gene plasmid
29 and treated with 50 nM acetyl-bufalin or the DMSO diluent. The results were normalized to the

30 Renilla luciferase activity. **C.** H460, A549 and PC-9 cells treated with concentration gradients of
31 acetyl-bufalin. STAT3 and its downstream target genes, including MCL-1, Survivin and VEGF,
32 were detected via western blot analysis. The relative intensity was calculated. *P<0.05, **P<0.01,
33 ***P<0.001, ****P<0.0001.

34

35 **Fig.S4 Chemistry experimental section of Bio-bufalin**

36 To a mixture of Biotin (0.3 mmol) and NEt₃ (0.45 mmol) in THF (dry, 3 mL) was added 2,4,6-
37 trichlorobenzoyl chloride (0.3 mmol), the reaction mixture was stirred at rt for 7 h. The bufalin (20
38 mg, 0.052 mmol), DMAP (0.4 mmol) and DCE (dry, 4 mL) were added. The suspension was stirred
39 at 100 °C for 2 h and then rt for 15 h. The mixture was then washed with 1 N HCl (aq, x 3) and
40 brine, purified by a silica gel flash chromatography (DCM/MeOH = 30:1 to 10:1) to afford target
41 compound (18.0 mg, 57% yield) as a white solid.

42 ¹H NMR (400 MHz, CDCl₃) δ 7.83 (dd, *J* = 9.6, 4.0 Hz, 1H), 7.23 (s, 1H), 6.26 (d, *J* = 9.6 Hz, 1H),
43 5.40 (s, 1H), 5.12 (s, 1H), 5.09 (s, 1H), 4.52-4.49 (m, 1H), 4.33-4.30 (m, 1H), 3.16-3.15 (m, 1H),
44 2.92 (dd, *J* = 12.8, 4.8 Hz, 1H), 2.74 (d, *J* = 12.8 Hz, 1H), 2.45-2.44 (m, 1H), 2.33 (t, *J* = 7.6 Hz,
45 2H), 2.20-2.17 (m, 1H), 2.09-1.94 (m, 1H), 1.96-1.81 (m, 2H), 1.80-1.35 (m, 24H), 1.21 (s, 3H),
46 0.70 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 173.0, 163.5, 162.3, 148.6, 146.7, 122.6, 115.3, 85.3,
47 70.3, 62.1, 60.2, 55.3, 51.3, 48.4, 42.4, 40.9, 40.5, 36.9, 35.9, 35.2, 34.3, 32.8, 30.6, 30.5, 29.7, 29.5,
48 28.7, 26.4, 25.5, 25.1, 23.8, 21.4, 21.3, 16.5; ESI-HRMS: calcd. for C₃₄H₄₉N₂O₆S⁺ (M+H)⁺
49 613.3306, found 613.3307.

50

51 **Fig.S5 CDK9 is a direct target of acetyl-bufalin**

52 **A.** Viability of PC-9 cells exposed to biotin-labeled bufalin as determined by MTT assay. **B.**
53 Representative image of an experimental microarray (blue = negative control, red = positive control,
54 yellow= positive spot). **C.** The expression of proteins involved in the CDK9/STAT3 pathway in
55 H460 and PC-9 cells was examined by Western blotting after acetyl-bufalin (Ac-bu) and SNS-032
56 (SNS) treatment, and the relative intensity was calculated. *p < 0.05, **p < 0.01, ***p < 0.00,
57 ****P<0.0001.

58

59 **Fig.S6 Acetyl-bufalin inhibited the CDK9/STAT3 signaling pathway**

60 **A.** CDK9 and STAT3 expression levels by western blotting after transduction with CDK9 siRNA
61 in H460 cells, the relative intensity was calculated. **B.** Western blot analysis of CDK9 and STAT3
62 expression levels in H460 and PC-9 cells transfected with CDK9 siRNA1 and treated for 30 min
63 with or without IL-6 (25 ng/ml). The relative intensity was calculated. *p < 0.05, **p < 0.01, ***p
64 <0.001, ****p<0.0001.

65

66 **Fig.S7 High CDK9 expression in human NSCLC tissues**

67 **A.** Protein expression of CDK9 in each paired human NSCLC tissue sample (T) and adjacent normal
68 lung tissues (N) from the same patient was detected by western blotting. **B.** Immunohistochemical
69 analysis for P-CDK9 from human NSCLC tissue samples and normal tissue samples.

70

71 **Supplementary Table 1:** IC50 of the main active components of cinobufagin in different NSCLC
72 cells.

73 **Supplementary Table 2:** The clinicopathologic characteristics of samples

74

Supplementary Table 1

Drug	IC₅₀ μM		
	PC-9	A549	H460
Arenobufagin	0.46	0.01274	
Bufalin	0.1409	0.08323	0.06472
Bufotalin	0.8133		
Pseudobufarenogin	> 100	4.268	
Cinobufagin	0.4144		
Cinobufotalin	0.5868	0.8186	
Desacetylcinobufagin	10.06	1.818	
Desacetylcinobufotalin	2.888	0.8867	
Gamabufotalin	0.281	0.7302	
Resibufogenin	4.64	1.129	1.061
Telocinobufagin	0.5741	0.1457	
Marinobufagin	15.00	1.547	1.301
Resibufagin	16.02		

Supplementary Table 2**The clinicopathologic characteristics of samples**

Number	Age	Sex	Pathological diagnosis	TNM
1	63	Male	Adenocarcinoma	T1bN0M0
2	59	Male	Squamous cell carcinoma	T3N0M0
3	52	Female	Adenocarcinoma	T1bN0MX
4	64	Female	Adenocarcinoma	T1cN1MX
5	71	Male	Adenocarcinoma	T2aN2M0
6	67	Female	Adenocarcinoma	T1bN0MX
7	74	Female	Squamous cell carcinoma	T4NXM0
8	66	Female	Adenocarcinoma	T1bN0M0
9	79	Female	Adenocarcinoma	T1cN0M0
10	68	Male	Adenocarcinoma	T1bN0M0
11	60	Male	Adenocarcinoma	T1bN0MX
12	54	Male	Adenocarcinoma	T1bN0M0