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Supplementary Material for:

Environment-Sensitive Turn-On Fluorescent Probes for p53-MDM2 Protein-Protein Interaction

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1. Materials and instruments

General chemicals available from commercial sources and used as received without further purified. The twice-distilled water used for the biological studies was used throughout all experiments. Buffer reagents were purchased from Aldrich and Acros. Melting points were measured on an electrothermal melting point apparatus. HRMS spectra were performed by the mass spectrometry facilities in the Shandong Analysis and Test Center. ^{1}H-NMR and ^{13}C-NMR were recorded on a Bruker 400 MHz NMR spectrometer. Fluorescence spectra and absorption spectra were got from a Thermo Varioskan microplate reader. Fluorescence imaging of cells was performed on a Zeiss Axio Observer A1 fluorescence microscope. Quantum yields of these probes were measured by HitachiF-2500 fluorescent spectrometer, Shimadzu UV-2401PC UV−visible spectrometer and WAY-2S Abbe refractometer. Analytical HPLC was performed on Agilent Technologies 1260 Series using a C8 reversed phase column (250 x 4.60mm , Phenomenex).

2. Docking study for probe 10 with MDM2

The initial three-dimensional geometric coordinates of the X-ray crystal structure of MDM2 (PDB code: 1T4E) was downloaded from the Protein Data Bank (http://www.pdb.org/pdb/home/home.do). Then we used GOLD 5.1with Gold Score fitness function to dock 3b into its targets for testing the binding conformation of the complex as we reported previously[1].

3. Synthesis

3.1 Characterization of 7-chlorobenzo[c] [1, 2, 5] oxadiazole-4-sulfonyl chloride (intermediate 3)

Chlorine acid (5 mL) was added in a 250 mL round bottom flask, 4-chlorobenzo[c] [1, 2, 5] oxadiazole (0.52 g, 3.36 mmol) was added in in portion wise and the mixture was heated at 120-130 °C for 6h. Then, the solution was cooled to room temperature. After cooling, the mixture was poured into ice slowly, and the precipitate was filtered and washed with water. The yellow product was obtained by recrystallizing from petroleum ether and benzene.

A yellow solid, Yield: 0.42 g, 71.0%. M.p.:87-88°C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.21-8.17(m, 1H), 7.68-7.66(m, 1H).

3.2 Characterization of 7-chloro-*N, N*-dimethylbenzo[c] [1, 2, 5] oxadiazole-4-sulfonamide (intermediate 4)

A solution of dimethylamine hydrochloride (76.8 mg, 0.94 mmol) and triethylamine (0.175 g, 1.738 mmol) in 10 mL THF was stirred for 0.5 h at room temperature. Then, 7-chloro-1, 3-dihydrobenzo[c] [1, 2, 5] oxadiazole-4-sulfonyl chloride (0.2 g, 0.79 mmol) was dissolved in 5 mL THF and added in slowly under ice bath. After 1h, the solution was removed under reduced pressure and extracted by dichloromethane, dried over $MgSO_4$ and evaporated *in vacuo.* The white product was obtained by column chromatography on silica gel (petroleum: dimethylamine = 2:1).

A white solid, Yield: 0.09 g. 43.7%. M.p.:142-143°C.^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.98 (d, *J* = 7.5Hz, 1H), 7.56 (d, *J* = 7.5Hz, 1H), 2.96 (s, 6H).

3.3 Characterization of 7-((2-aminoethyl) amino)-*N, N*-dimethylbenzo[c] [1, 2, 5] oxadiazole-4-sulfonamide (intermediate 5)

A solution of ethylenediamine (1 mL) and compound **4** (50.0 mg, 0.19 mmol) in 4 mL acetonitrile was stirred for 6 h at 60 °C. Then, the solvent was removed under reduced pressure, and the yellow product **5** was obtained by column chromatography on silica gel (dichloromethane: methanol = 10:1).

A yellow solid, Yield: 0.05 g. 92.3%. M.p.:132-135°C.^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.90(d, *J* = 6.0 Hz, 1H), 6.25(s, 1H), 6.15(d, *J* = 6.0 Hz, 1H), 3.45-3.39(m, 2H), 3.14-3.10(m, 2H), 2.87(s, 6H), 1.31(s, 2H).

3.4 Characterization of 7-((3-aminopropyl) amino)-*N, N*-dimethylbenzo[c] [1, 2, 5] oxadiazole-4-sulfonamide (intermediate 6)

A yellow solid, Yield: 0.11 g. 64.7%. M.p.:108-110°C.^{1}HNMR ($CDCl_3$, 400 MHz): δ 7.83(d, *J* = 9.0 Hz, 1H), 6.32(d, *J* = 9.0 Hz, 1H), 4.12(s, 2H), 3.45(t, *J* = 6.0 Hz, 2H), 2.69-2.65(m, 8H), 1.79-1.68(m, 2H).

3.5 Characterization of 7-((aminomethyl) amino)-*N, N*-dimethylbenzo[c] [1, 2, 5] oxadiazole-4-sulfonamide (intermediate 7)

A yellow solid, Yield: 0.12 g. 75.0%. M.p.:115-118°C.^{1}HNMR ($CDCl_3$, 400 MHz): δ 7.83(d, *J* = 6.0 Hz, 1H), 6.31(d, *J* = 9.0 Hz, 1H), 4.44(s, 2H), 3.38(t, *J* = 6.0 Hz, 2H), 2.69(s, 6H), 2.61-2.56(m, 2H), 1.77-1.40(m, 3H).

3.6 Characterization of 7-((2-((1-(3-(*1H*-imidazol-1-yl)propyl)-4-benzoyl-5-(4-bromophenyl)-2-oxo-2,5-dihydro-*1H*-pyrrol-3-yl)amino) ethyl) amino)-*N, N*-dimethylbenzo[c][1,2,5]oxadiazole-4-sulfonamide(probe 9)

Compound **8** (40.0 mg, 0.09 mmol) and intermediate **5** (77.0 mg, 0.3 mmol) were added to a tube. Then, 1.5 mL acetic acid was added and the mixture reacted under microwave. Two hours later, the solvent was removed and the product **9** was got by column chromatography on silica gel (petroleum: dichloromethane = 33:1).

A yellow solid, Yield: 0.01 g. 12.3%. M.p.:115-117°C. ^{1}H-NMR (300 MHz, DMSO-d_6),δ 9.25(brs, 1H), 8.62-8.59(m, 1H), 7.82(d, *J* = 6.0 Hz, 1H), 7.58(s, 1H), 7.38-7.19(m, 7H), 7.12(s, 1H), 6.87(s, 1H), 6.71(d, *J* = 6.0 Hz, 2H), 6.46(d, *J* = 6.0 Hz, 1H), 4.14(brs, 1H), 4.04-3.80(m, 2H), 3.64(s, 2H), 3.50-3.34(m, 2H), 2.66(s, 6H), 1.84-1.75(m, 2H). ^{13}C-NMR ($CDCl_3$, 75 MHz):δ 191.58, 165.50, 146.44, 144.58, 140.88, 139.94, 139.90,137.27, 134.81, 131.73, 130.67, 129.46, 129.10, 128.14, 126.62 , 122.37, 118.58, 112.28, 109.20, 98.98, 62.55, 44.79, 40.67, 38.53, 37.82, 29.69. HRMS (ESI) m/z calcd.for $C_{33}H_{34}BrN_8O_5S$ ([M + H] $^{+}$) 733.1556; found 733.1558.

3.7 Characterization of 7-((3-((1-(3-(*1H*-imidazol-1-yl)propyl)-4-benzoyl-5-(4-bromophenyl)-2-oxo-2,5-dihydro-*1H*-pyrrol-3-yl) amino)propyl)amino)-*N,N*-dimethylbenzo[c][1,2,5]oxadiazole-4-sulfonamide(probe 10)

A yellow solid, Yield: 0.07 g. 10.0%. M.p.:108-110°C. ^{1}H-NMR (300 MHz, DMSO-d_6), δ 8.97(brs, 1H), 8.47(s, 1H) ,7.80(d, *J* = 6.0 Hz, 1H), 7.57(s, 1H), 7.36-7.12(m, 7H), 7.12(s, 1H), 6.86(s, 1H), 6.77(d, *J* = 9.0 Hz, 2H), 6.32(d, *J* = 9.0 Hz, 1H), 5.56(s, 1H), 3.92-3.83(m, 3H), 3.45-3.41(m, 2H), 3.34(s, 1H), 2.67(s, 6H),1.98(s, 1H),1.83-1.78(m, 2H),1.24(s, 1H),0.85-0.83(m, 1H). ^{13}C-NMR ($CDCl_3$, 75 MHz):δ 191.89, 165.15, 148.91, 146.42, 144.56, 140.95, 140.35,139.62, 137.25, 135.08, 131.75, 130.42, 129.46, 129.00, 128.23, 126.36, 122.31, 118.44, 112.12, 108.93, 98.63, 62.64, 44.88, 40.52,40.06, 38.68, 37.84, 29.77,29.09. HRMS (ESI) m/z calcd.for $C_{34}H_{36}BrN_8O_5S$ ([M + H] $^{+}$) 747.1713; found 747.1718.

3.8 Characterization of 7-((((1-(3-(*1H*-imidazol-1-yl)propyl)-4-benzoyl-5-(4-bromophenyl)-2-oxo-2,5-dihydro-*1H*-pyrrol-3-yl)amino)methyl)amino)-*N,N*-dimethylbenzo[c][1,2,5]oxadiazole-4-sulfonamide(probe 11)

A yellow solid, Yield: 0.05 g. 7.4%. M.p.:98-100°C. ^{1}H-NMR (300 MHz, DMSO-d_6), δ 9.01(brs, 1H), 8.47(s, 1H), 7.81(d, *J* = 6.0 Hz, 1H), 7.57(s, 1H), 7.36-7.23(m, 7H), 7.11(s, 1H), 6.85(s, 1H), 6.77(d, *J* = 6.0 Hz, 2H), 6.32(d, *J* = 9.0 Hz, 1H), 5.57(s, 1H), 3.94-3.81(m, 4H), 3.50-3.42(m, 2H), 3.33(s, 1H), 2.67(s, 6H), 1.98(s, 1H), 1.84-1.80(m, 2H), 1.68(s, 4H). ^{13}C-NMR ($CDCl_3$, 75 MHz):δ 191.80, 165.11, 146.41, 144.50, 140.83, 140.46,139.66, 137.09, 135.28, 131.74, 130.36, 129.51, 129.00, 128.21, 126.38, 122.26, 118.50, 111.73, 109.04, 98.73, 62.67, 44.77, 42.64, 41.58, 38.67, 37.83, 29.70,29.15,25.28. HRMS (ESI) m/z calcd.for $C_{35}H_{38}BrN_8O_5S$ ([M + H] $^{+}$) 761.1869; found 761.1866.

4. Fluorescence spectroscopy test of probes 9-11.

Probe was dissolved in DMSO to acquire 10mM stock solution, respectively. The stock solution of them was diluted in PBS (PH =7.4) to obtain 5 μM solutions. The fluorescent properties of them were obtained on Thermo-Fisher Varioskan microplate reader. The quantum yields of them were calculated under PBS solution (pH = 7.4) and DCM solution, respectively, using fluorescein 0.1 M NaOH (Φ_{ST} = 0.92) as a reference.

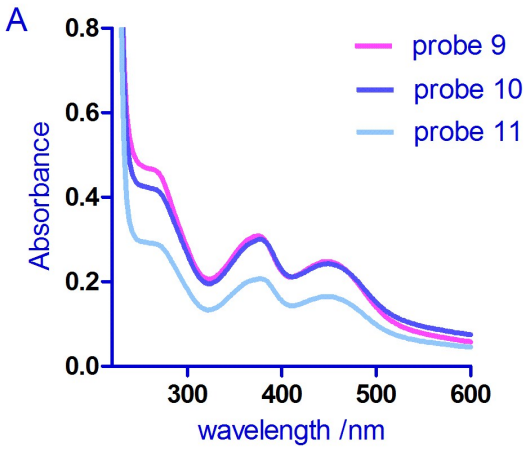


Fig.S1 Absorption spectra of probes **9-11**.

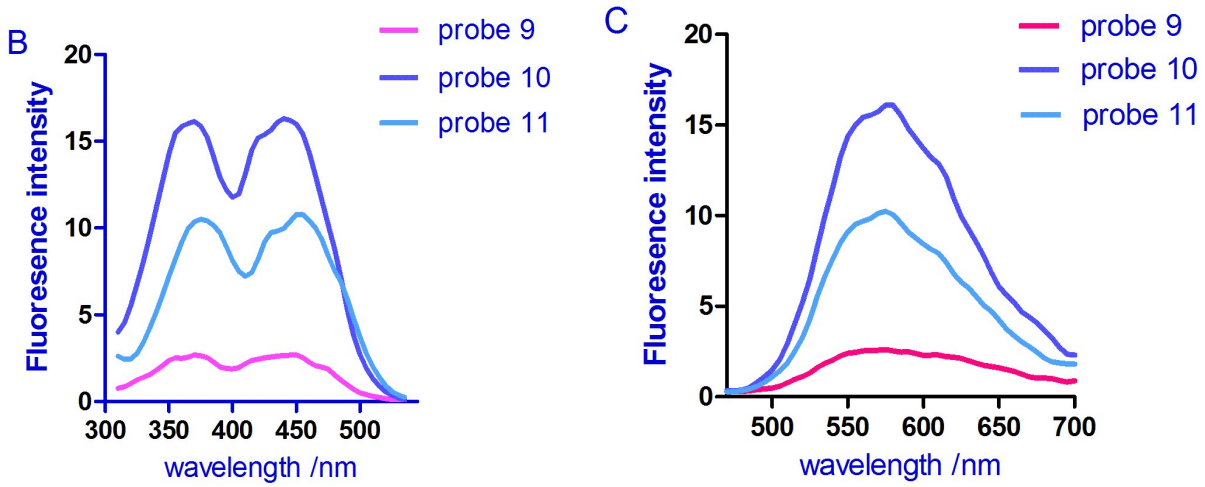


Fig.S2 (A) Fluorescence spectroscopy of probes **9, 10,** and **11**; (B) fluorescence excitation spectrum and (C) fluorescence emission spectrum.

5. The effect of probes 9-11 to the FP assay

The direct fluorescence response of probe **9-11** to MDM2–p53 was determined on a Multiskan Spectrum Microplate Spectrophotometer. Various concentrations of MDM2 protein at the range of 0.0-0.25 μM were preincubated with p53 peptide (10 nM; ChinaPeptides Co., Ltd.,Shanghai, China) for 30 min. Then, probe **9-11** was added to the mixture, and the final concentration of the probe is 10 μM. After 5–10 min, the emission spectrums at different protein concentration were obtained, which is excited at 440 nm, and we then analyzed the fluorescent intensity at the maximum emission wavelength 543 nm with the changes of MDM2–p53 protein concentration.

Table S1 FP value changes of probes **9-11** (100 nM) with increased MDM2 protein concentration using the similar method of FP assay.

MDM2 (μM)	0.033725	0.0675	0.125	0.25
9	132.5±0.71	131.5±10.08	140.0±57.30	124.5±15.20
10	130.0±7.50	133.5±3.70	136.5±23.45	132.0±16.30

11	134.5±11.20	126.5±1.80	137.5±10.80	124.0±15.50

6. MDM2 binding affinity assay

Briefly, the fluorescence polarization experiments were read on BioTek (Winooski, VT, USA) Synergy H4 with the 485-nm excitation and 535-nm emission filters. The fluorescence intensities parallel (Intparallel) and perpendicular (Intperpedicular) to the plane of excitation were measured in black 96-well NBS assay plates (Corning # 3993) at room temperature. The background fluorescence intensities of blank samples containing the reference buffer were subtracted, and steady-state fluorescence polarization was calculated using the following equation: P = 1000 9 (Intparallel-GIntperpedicular)/(Intparallel + GIntperpedicular), and the correction factor G (G = 0.998 determined empirically) was introduced to eliminate differences in the transmission of vertically and horizontally polarized light. All fluorescence polarization values were expressed in millipolarization units (mP). The dose-dependent binding experiments were carried out with serial dilution in DMSO of compounds. A 5 μL sample of the test sample preincubated (for 30 min) with MDM2 binding domain (1–118) (10 nM) and PMDM-F peptide (Anaspec) (10 nM) in assay buffer (100 mM potassium phosphate, pH 7.5; 100 mg/mL bovine gamma globulin; 0.02% sodium azide) were added to microplates to produce a final volume of 115μL. Plates were read at 1 h after mixing all assay components. Binding constant (K_i) and inhibition curves were fitted using GraphPad Prismsoftware. Nutlin-3a (Sigma-Aldrich, St. Louis,MO, USA) was used as a reference compound for validating the assay in each plate.

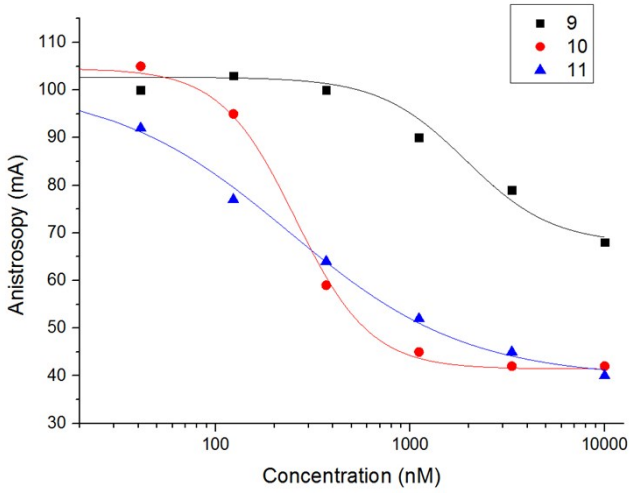


Fig.S3 Competitive binding curves of probes **9-11** to MDM2 by FP assay

7. Fluorescent emission spectra of probes 9-11 combined with MDM2, BSA

Using similar protocol, we choose bovine serum albumin (BSA) which often forms non-specific binding with small molecule and MDM2 protein as the control groups to evaluate the selectivity of probe **9-11** to MDM2-p53. The concentration of BSA and MDM2 is identical to MDM2-p53 and then we analyzed the fluorescent intensity at emission wavelength 543 nm with the changes of protein concentration, which is excited at 440 nm.

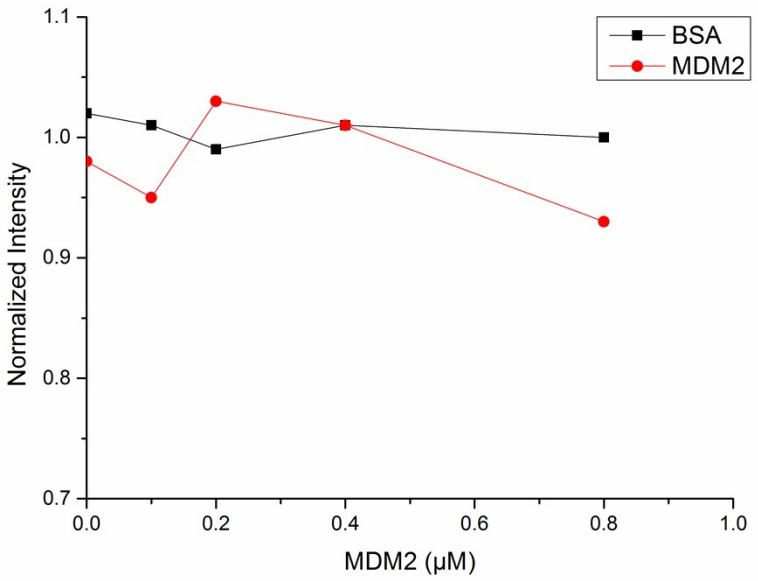


Fig.S4 The fluorescent properties of probes **9-11** at the emission wavelength 543 nm with a series of concentrations of BSA and MDM2 (0.86 – 0 μM), excited at 440 nm.

8. *In vitro* antiproliferative assay of probes 9-11

The cellular growth inhibitory activity of probes was determined by SRB (Sulforhodamine B) assays, using A549 (wild-type p53), H1299 (p53 null), HCT116 (wild-type p53), and MDA-MB-231 (wild-type p53) cells. An amount of (4-6)×10^3 cells per well were placed in 96-well plates with 100 μL culture medium and then cultured in 5% CO2 atmosphere at 37℃ for 12 h. Subsequently, the test compounds were added to the wells at different concentrations for 48 h. Then, 100 μL cold 10% (wt/vol) TCA (Trichloroacetic acid) was added to each well gently and incubated at 4℃. After 1 h, the plates were washed by water purified with a Mill-Q filtration system four times and excess water was removed using paper towels. Then, the plates were dried at room temperature. Once dry, 100μL of 4 g/L SRB solution was added to each well. After shaking for 30 min, the plates were rinsed by 1 %(vol/vol) acetic acid four times to remove unbound dye and dried at room temperature. Then, 100μL of 10 mM Tris base solution (PH 10.5) were added to each well. After shaking for 10 min, the absorbance value of each well was recorded using a microplate reader at 515 nm. Wells containing no compounds were chosen as blanks. The concentration of each probe that inhibited cell growth by 50% (IC_{50}) was calculated.

Table S2 *In vitro* antiproliferative activities of compounds

Compd.	A549	NCI-H1299	HCT116	MDA-MB-231
	100 μM Inhibitory Rate %			
9	53.4	42.5	50.0	40.6
10	37.3	39.8	45.4	50.6
11	3.3	40.4	35.3	34.6

9. Fluorescence microscopy imaging.

Cell Culture. The fluorescent imaging was performed on two human lung cancer cell lines [A549 (wild-type p53) and NCI-H1299 (p53 null)].They were cultured in RPMI-1640 medium which was added with 10% (v/v) fetal bovine serum

under an atmosphere of 5% CO_2 and 95% air at 37 °C. When the cells have proliferated sufficiently, these two types of cells were placed on a confocal dish for 12–24 h.

***Staining*.** After the culture medium had been removed, cells were washed with RPMI-1640 medium without 10% (v/v) fetal bovine serum carefully and incubated at room temperature. Probes **9-11** were dissolved in DMSO to get a stock solution (10 mM). Then, the solution was further diluted with RPMI-1640 medium without fetal bovine serum. Subsequently, probe**s** were incubated with A549 and NCI-H1299 cells for 15 min, respectively.

The imaging was also performed by the positive control (100 μM Nutlin-3) incubation together with 5 μM each probe at the same conditions, using A549 and NCI-H1299 cells.

Fluorescence imaging. Fluorescence imaging in live A549 (wild-type p53) and NCI-H1299 cells (p53 null) were obtained on a Zeiss Axio Observer A1 fluorescent microscope. Objective lens: 63×.

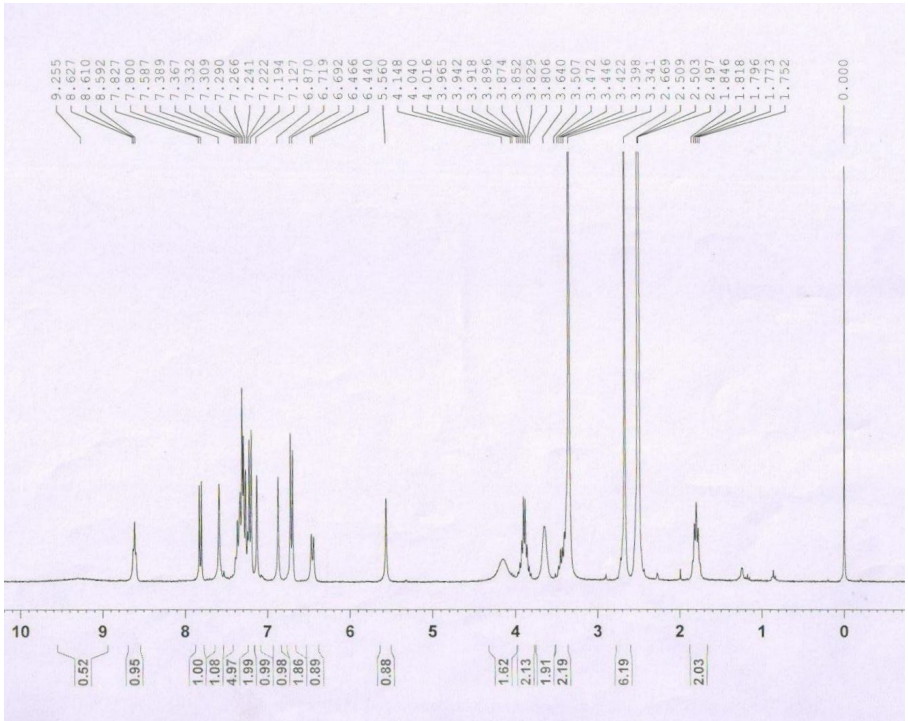
10. Flow cytometry analysis.

The flow cytometry test was performed on A549 cells. 6×10^5 cells were collected and washed with 10 mM PBS for at least three times until the culture medium was cleared completely. Probe **9-11** with the final concentration of 5 μM was added respectively or together with the positive control (100 μM Nutlin-3) into the flow tubes with 250 μL PBS and 1×10^5 cells each tube. After incubation for about half an hour at 37 °C, the samples could be analyzed by BD FACSCalibur.

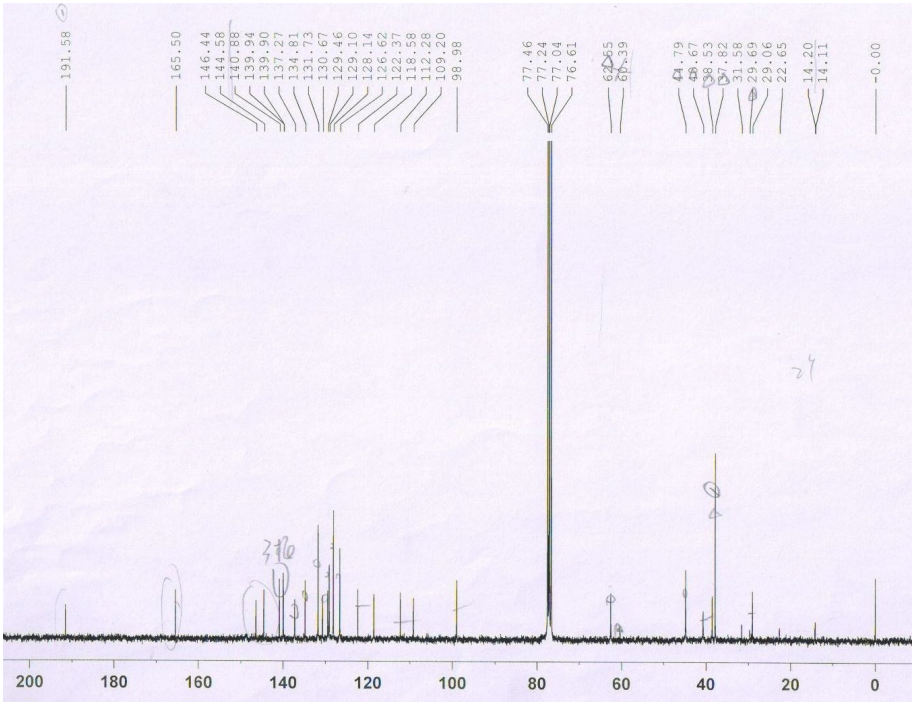
11. References

1. C. Zhuang, Z. Miao, Y. Wu, Z. Guo, J. Li, J. Yao, C. Xing, C. Sheng and W. Zhang, *J. Med. Chem.,* 2014, **57** , 567-577.

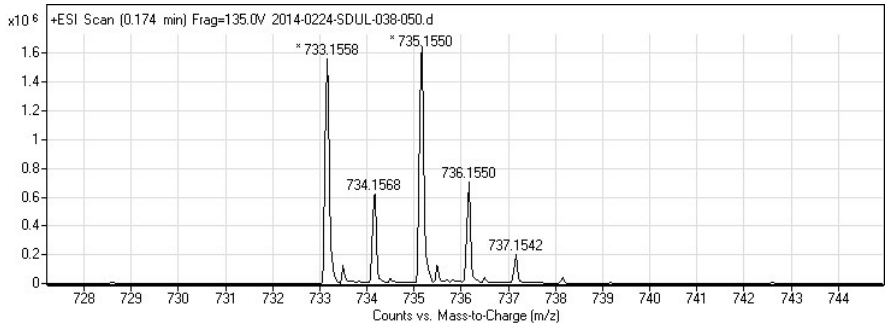
12. Charaterization of probes



^{1}H-NMR spectrum of compound **9**.



^{13}C-NMR spectrum of compound **9**.



HRMS(ESI) of compound **9**

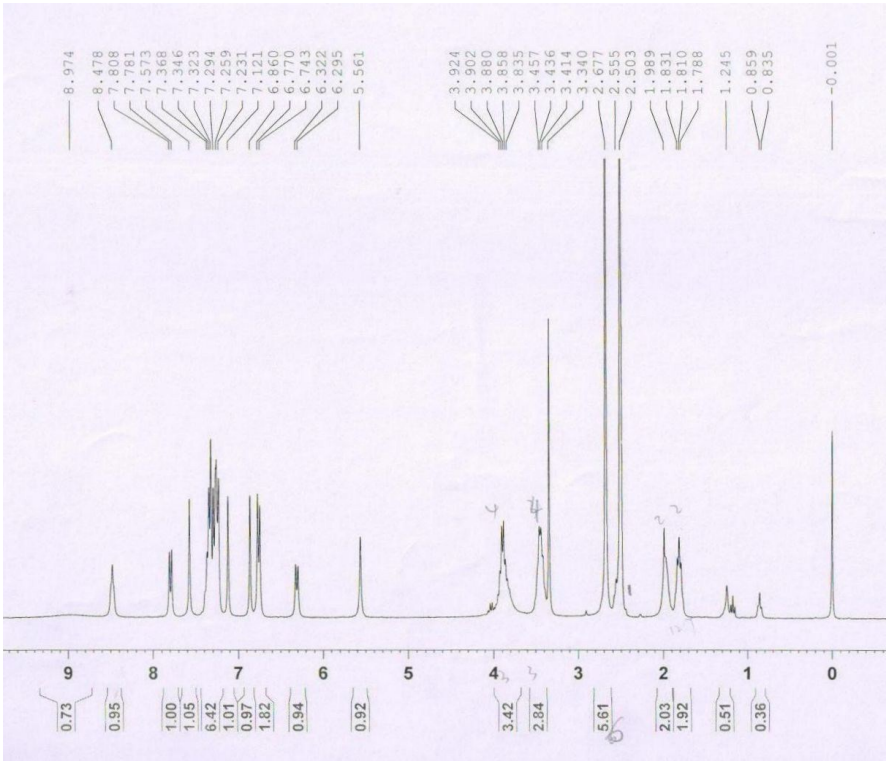
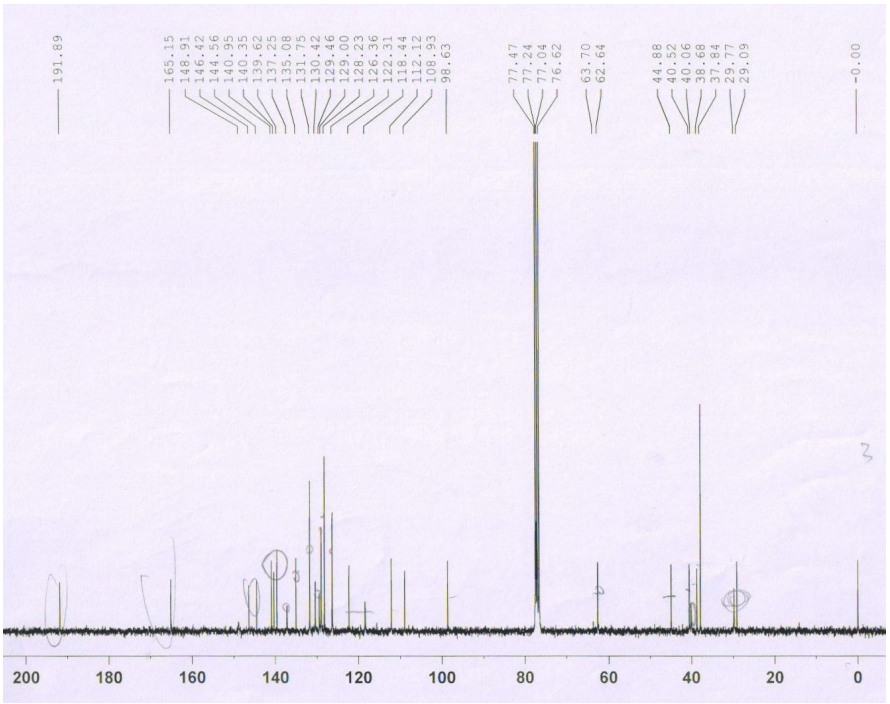
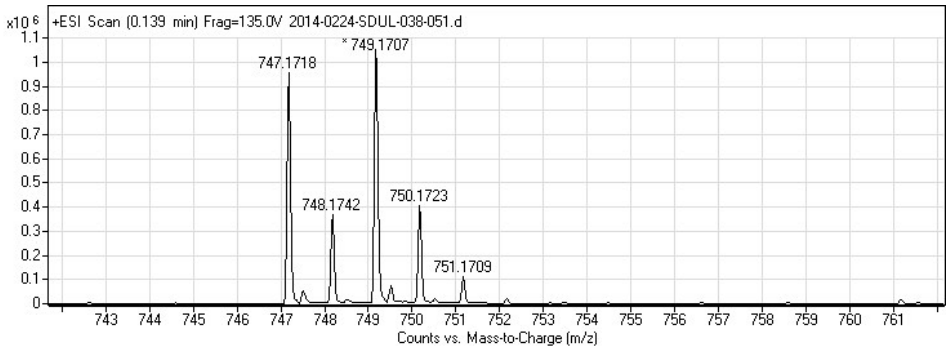


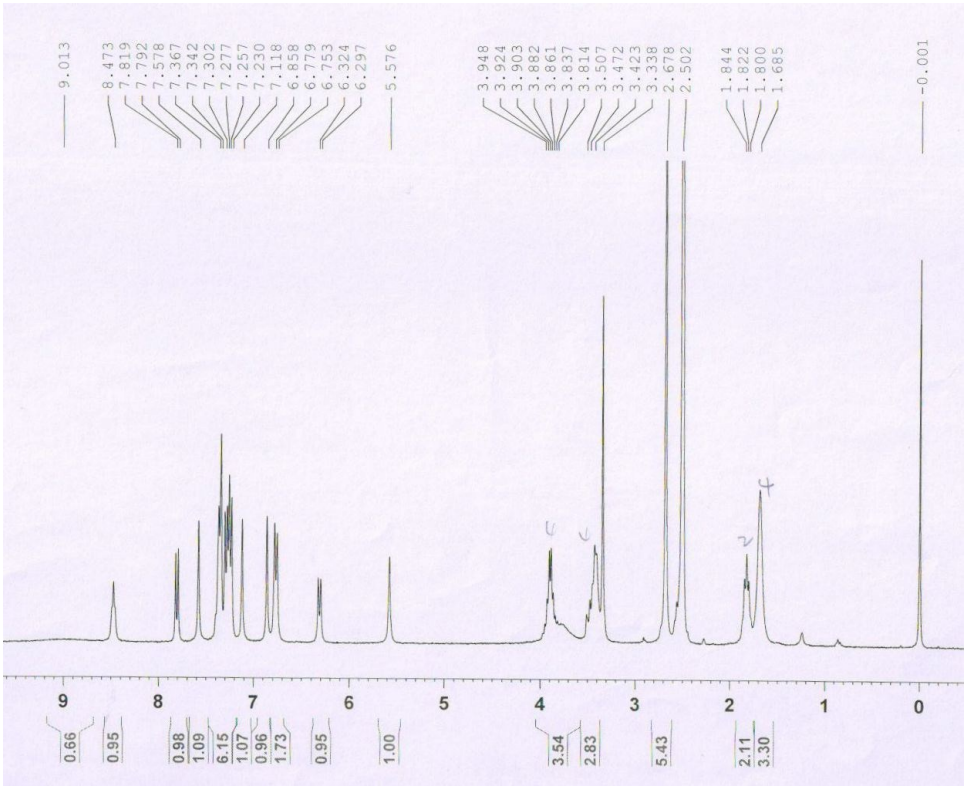
Figure S12 ^{1}H-NMR spectrum of compound **10**.



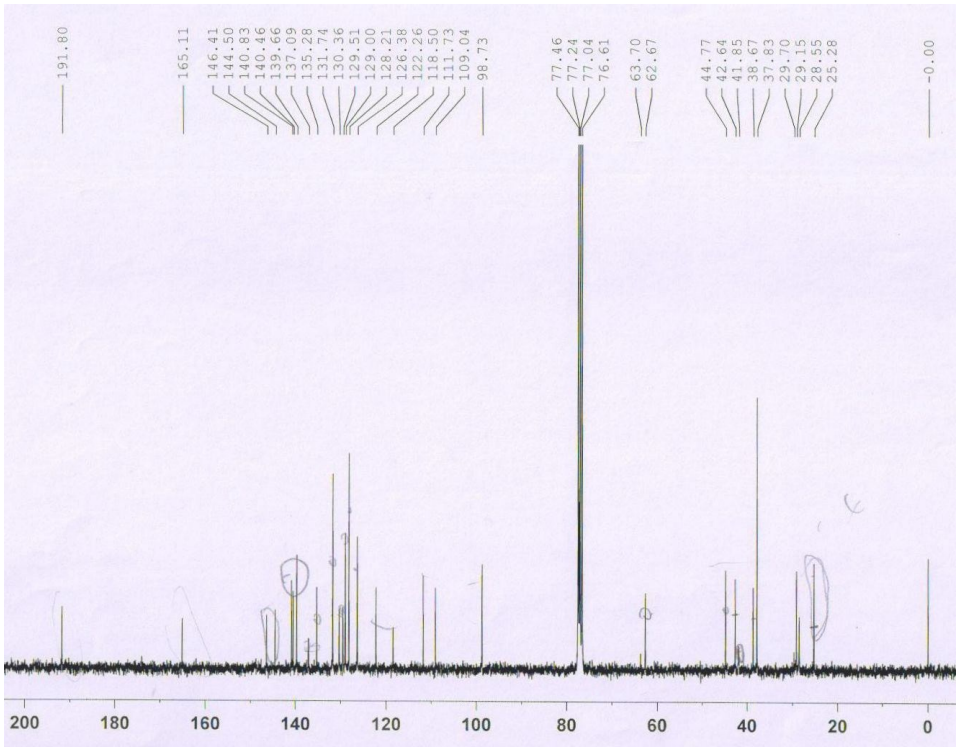
^{13}C-NMR spectrum of compound **10**.



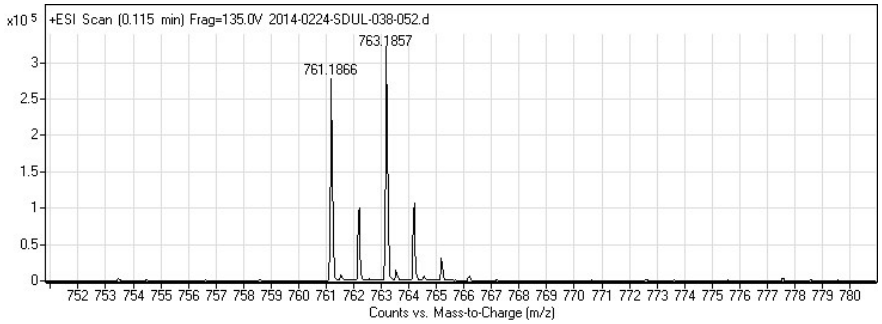
HRMS(ESI) of compound **10**.



^{1}H-NMR spectrum of compound **11**.



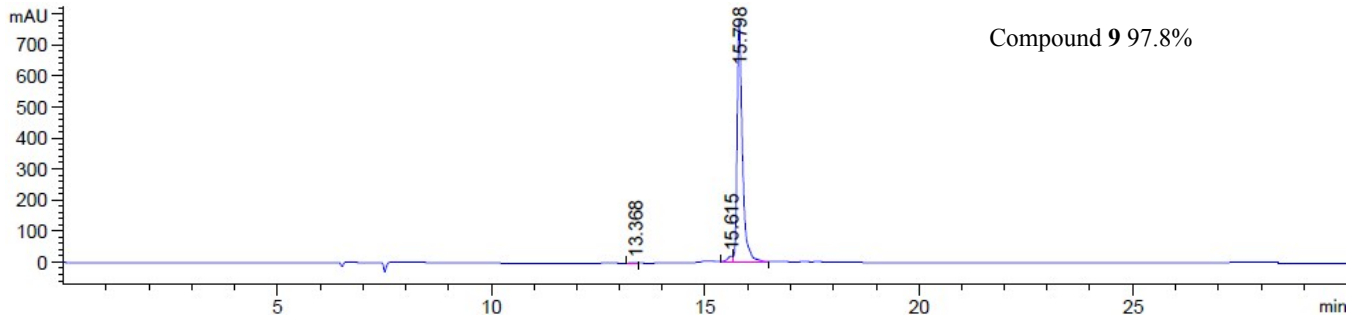
^{13}C-NMR spectrum of compound **11**.



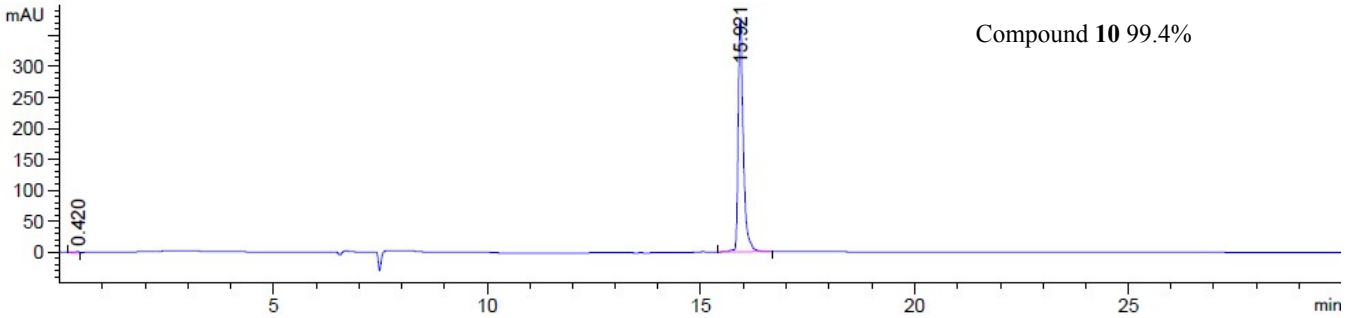
HRMS(ESI) of compound **11**.

HPLC assessment of the products purity.

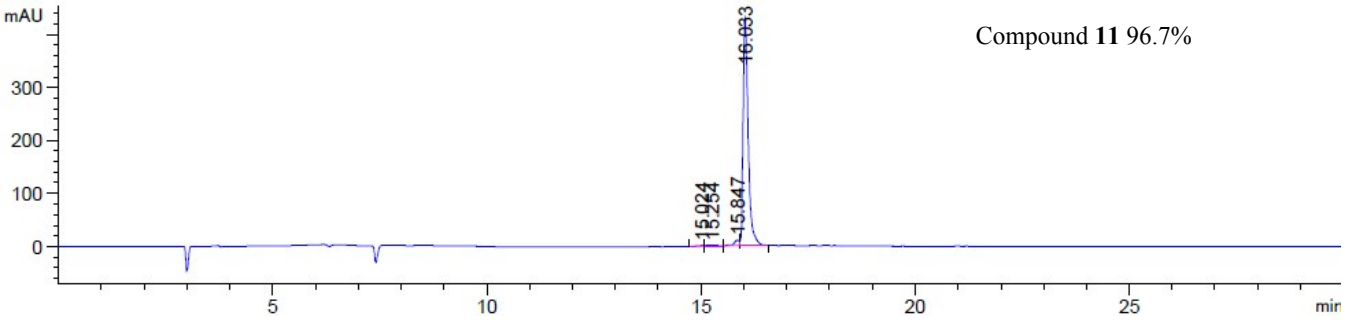
All the compounds with a purity of >95% was used for subsequent biological assays. Moreover, we provided the spectra of HPLC assay as below.



Compound **9** 97.8%



Compound **10** 99.4%



Compound **11** 96.7%