Enhancing the solubility and bioactivity of anticancer drug tamoxifen by water-soluble pillar[6]arene-based host–guest complexation

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1. Materials and methods

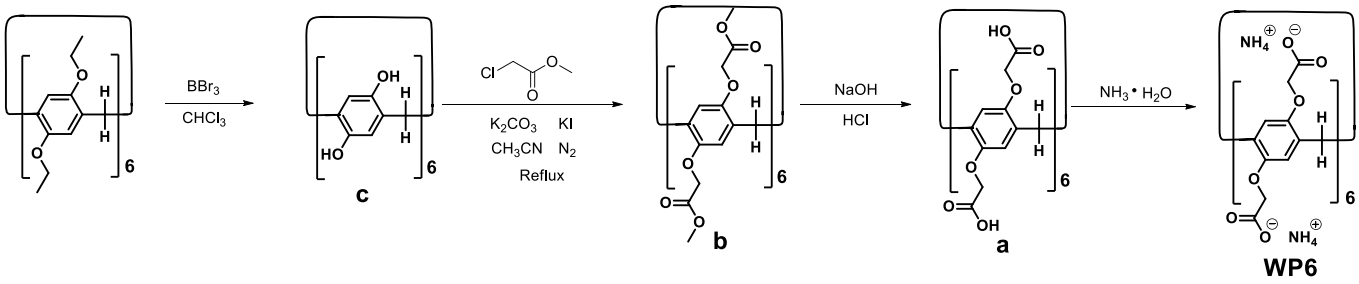
General methods: All reagents were commercially available and used as supplied without further purification. NMR spectra were recorded with a Bruker Avance DMX 400 spectrophotometer or a Bruker Avance DMX 400 spectrophotometer with use of the deuterated solvent as the lock and the residual solvent or TMS as the internal reference. Low-resolution electrospray ionization mass spectra were obtained on a Bruker Esquire 3000 plus mass spectrometer (Bruker-Franzen Analytik GmbH Bremen, Germany) equipped with an ESI interface and an ion trap analyzer. UV–vis spectra were taken on a Perkin-Elmer Lambda 35 UV–vis spectrophotometer. The fluorescence experiments were conducted on a RF-5301 spectrofluorophotometer (Shimadzu Corporation, Japan).

Cell culture: MCF-7 and Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells grew as a monolayer and were detached upon confluence using trypsin (0.5% w/v in PBS). The cells were harvested from the cell culture medium by incubating in a trypsin solution for 5 min. The cells were centrifuged, and the supernatant was discarded. A 3 mL portion of serum-supplemented DMEM was added to neutralize any residual trypsin. The cells were resuspended in serum-supplemented DMEM at a concentration of 1×10^4 cells/mL. Cells were cultured at 37 °C and 5% CO_2.

Evaluation of cytotoxicity: The cytotoxicity of **WP6**, **WP6-T**, and free drug tamoxifen against MCF-7 and Hela cells were determined by 3-(4',5'-dimethylthiazol-2'-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays in a 96-well cell culture plate. All solutions were sterilized by filtration with a 0.22 μm filter before tests. MCF-7 and Hela cells were seeded at a density of 1×10^4 cells/well in a 96-well plate, and incubated for 24 hours for attachment. Cells were then incubated with **WP6**, **WP6-T**, and free drug tamoxifen at various concentrations for 4 hours or 12 hours. The sample of **WP6-T** was prepared by dissolving tamoxifen in a 5.00 mM **WP6** aqueous solution and then diluted to desired concentrations. The free drug tamoxifen was dissolved in DMSO as a concentrated solution for the poor solubility of tamoxifen in water. Afterwards, it was diluted to desired concentrations in the condition of keeping the content of DMSO lower than 5%. After washing the cells with PBS buffer, 20 μL of a MTT solution (5 mg/mL) was added to each well. After 4 hours of incubation at 37 °C, the MTT solution was removed, and the insoluble formazan crystals that formed were dissolved in 100 μL of dimethylsulfoxide (DMSO). The absorbance of the formazan product was measured at 570 nm using a spectrophotometer (Bio-Rad Model 680). Untreated cells in media were used as a control. All experiments were carried out with three replicates.

*2. Synthesis of compound **WP6***

***Scheme S1.** Synthetic route to **WP6**.*



Compound **WP6** was synthesized according to literature procedures.[S1,S2,S3] The proton NMR spectrum of **WP6** is shown in Fig. S1. ^{1}H NMR (400 MHz, $CDCl_3$, 298 K) δ (ppm): 6.62 (s, 12H), 4.10 (s, 24H), 3.84 (s, 12H).

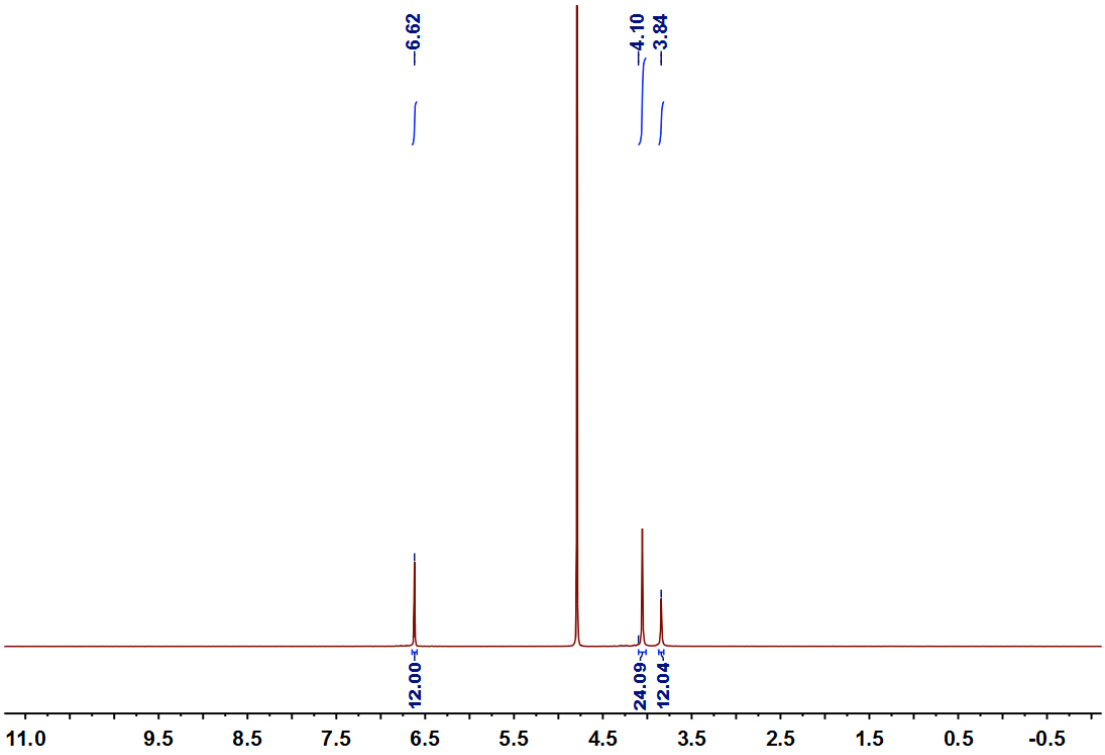


Figure S1. ^{1}H NMR spectrum (400 MHz, D_2O, 298 K) of **WP6**.

*3. ^{1}H NMR spectra recorded for the solubility determination of **WP6***

Compound **WP6** was added in excess to 1.00 mL of deuterium oxide. The resultant suspension was magnetically stirred at room temperature for 24 hours. After that, the undissolved **WP6** was removed by filtration (0.22 μm) to obtain the saturated solution of **WP6**. Certain amount of internal reference sodium benzene-1,3,5-tricarboxylate was added into 0.50 mL of the saturated solution and the ^{1}H NMR spectrum was recorded. The concentration of **WP6** in the solution was measured by comparing the integration of a known concentration of sodium benzene-1,3,5-tricarboxylate as internal standard with selected ^{1}H NMR resonances of **WP6**. The measurements were carried out with three replicates and the average was supposed to be the solubility of **WP6**.

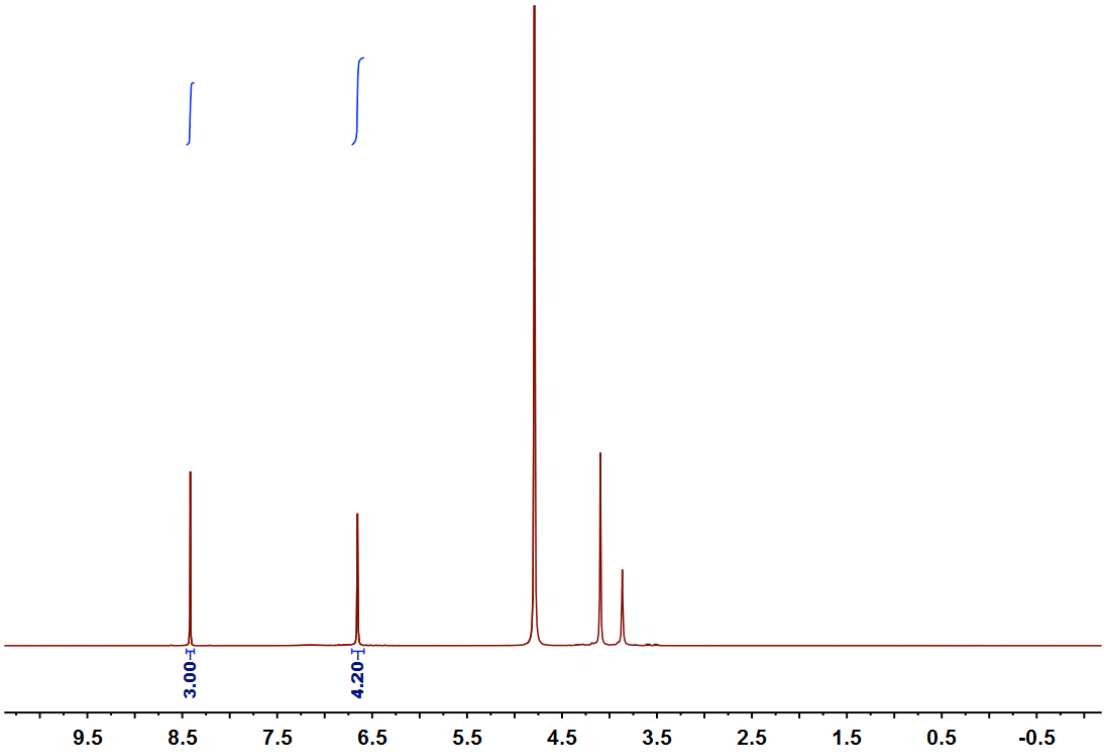


Figure S2. Partial ^{1}H NMR spectrum (400 MHz, 298 K) of the saturated solution of **WP6** in D_2O added with 21.85 mg of sodium benzene-1,3,5-tricarboxylate as the internal reference. The concentration of **WP6** was calculated to be 55.4 mM.

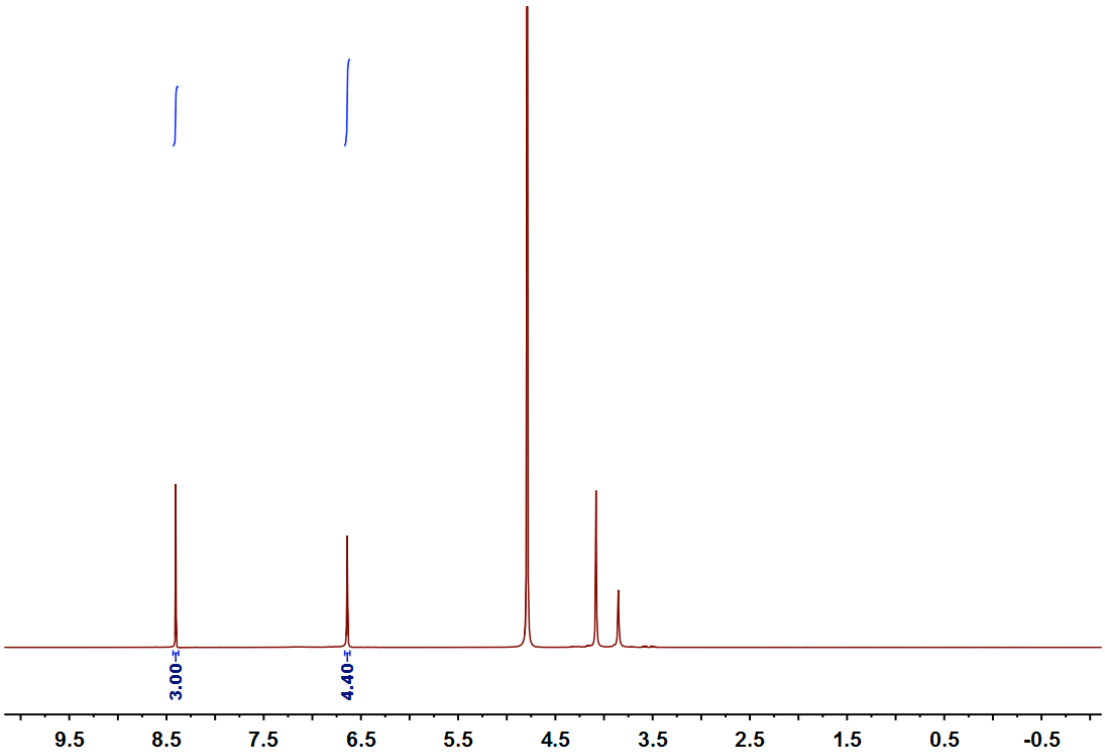


Figure S3. Partial ^{1}H NMR spectrum (400 MHz, 298 K) of the saturated solution of **WP6** in D_2O added with 21.67 mg of sodium benzene-1,3,5-tricarboxylate as the internal reference. The concentration of **WP6** was calculated to be 57.6 mM.

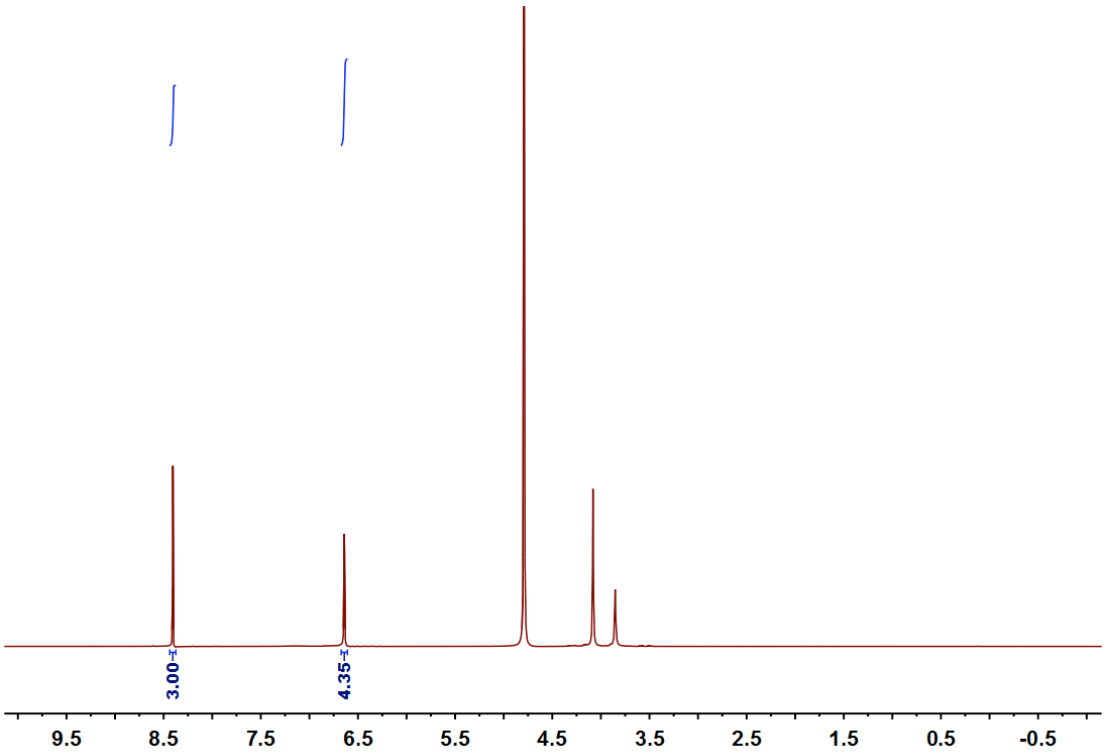


Figure S4 Partial ^{1}H NMR spectrum (400 MHz, 298 K) of the saturated solution of **WP6** in D_2O added with 20.96 mg of sodium benzene-1,3,5-tricarboxylate as the internal reference. The concentration of **WP6** was calculated to be 56.8 mM.

4. Partial NOESY NMR spectrum of ***WP6-T***

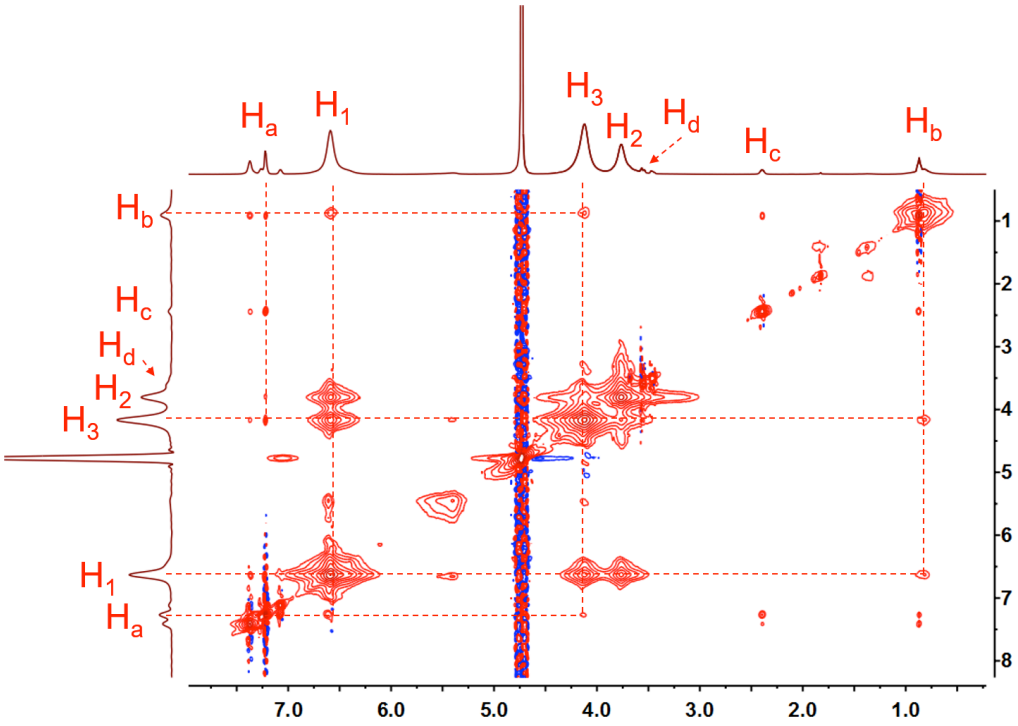


Figure S5. Partial 2D NOESY NMR spectrum (500 MHz, D_2O, 298 K) of tamoxifen dissolved in the solution of **WP6** (20.0 mM).

5. Electrospray ionization mass spectrum of ***WP6-T***

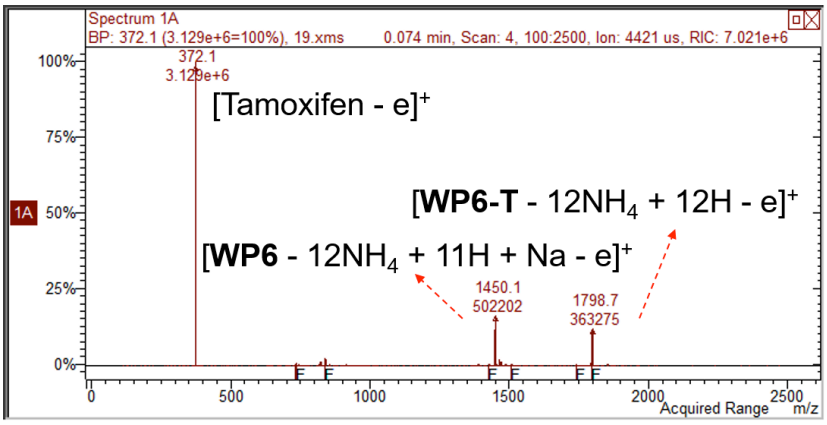


Figure S6. Electrospray ionization mass spectrum of the sample of tamoxifen dissolved in the solution of **WP6** (5.00 mM).

6. ^{1}H NMR spectra recorded for phase solubility diagram

Into the solutions of **WP6** in D_2O which were prepared with concentrations ranging from 1.00 mM to 50.0 mM, excess amount of pharmaceutical agent tamoxifen was added and magnetically stirred at room temperature for 24 hours. After that, the excessive undissolved tamoxifen was removed by filtration (0.22 μm) to obtain the sample solutions. Certain amount of internal reference sodium benzene-1,3,5-tricarboxylate was added into 0.50 mL of each of the sample solutions and the ^{1}H NMR spectrum was recorded. The concentration of tamoxifen in the supernatant solution was measured by ^{1}H NMR (400 MHz) spectroscopy by comparing the integration of a known concentration of sodium benzene-1,3,5-tricarboxylate as the internal standard with the selected ^{1}H NMR resonances of tamoxifen.

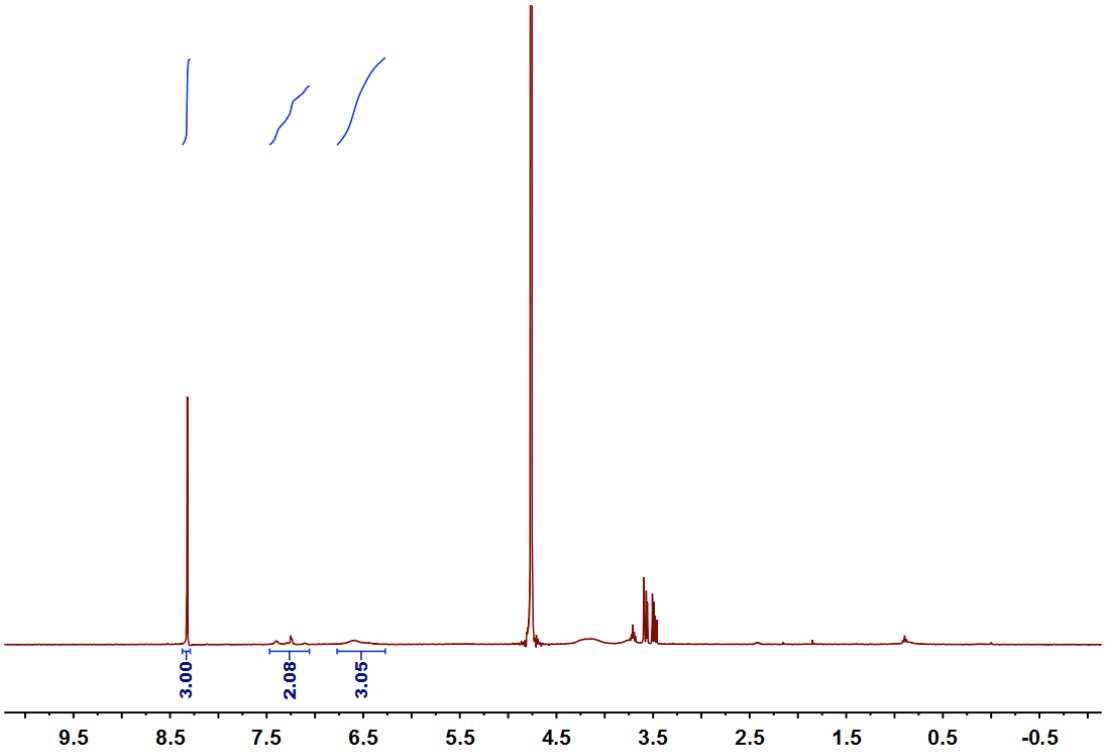


Figure S7. ^{1}H NMR (400 MHz, 298 K, D_2O) spectrum recorded for tamoxifen dissolved in a solution of **WP6** (0.50 mL,1.00 mM). Here 0.90 mg of sodium benzene-1,3,5-tricarboxylate was added as the reference.

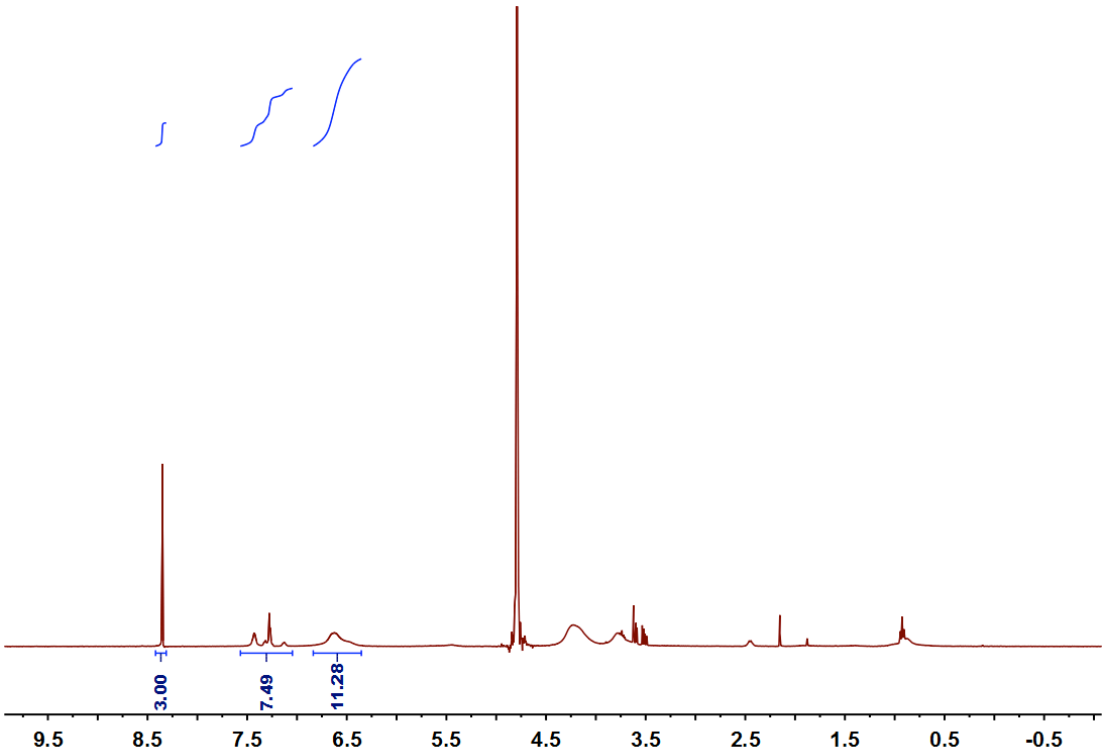


Figure S8. ^{1}H NMR (400 MHz, 298 K, D_2O) spectrum recorded for tamoxifen dissolved in a solution of **WP6** (0.50 mL, 5.00 mM). Here 1.04 mg of sodium benzene-1,3,5-tricarboxylate was added as the reference.

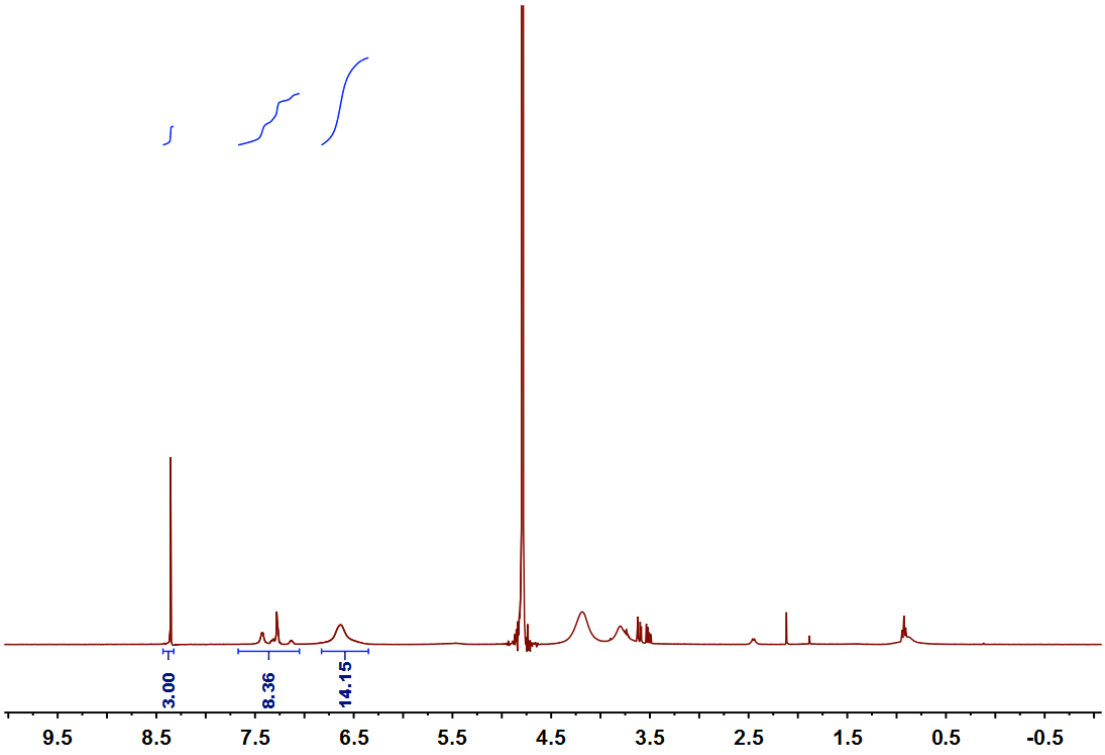


Figure S9. ^{1}H NMR (400 MHz, 298 K, D_2O) spectrum recorded for tamoxifen dissolved in a solution of **WP6** (0.50 mL, 10.0 mM). Here 1.10 mg of sodium benzene-1,3,5-tricarboxylate was added as the reference.

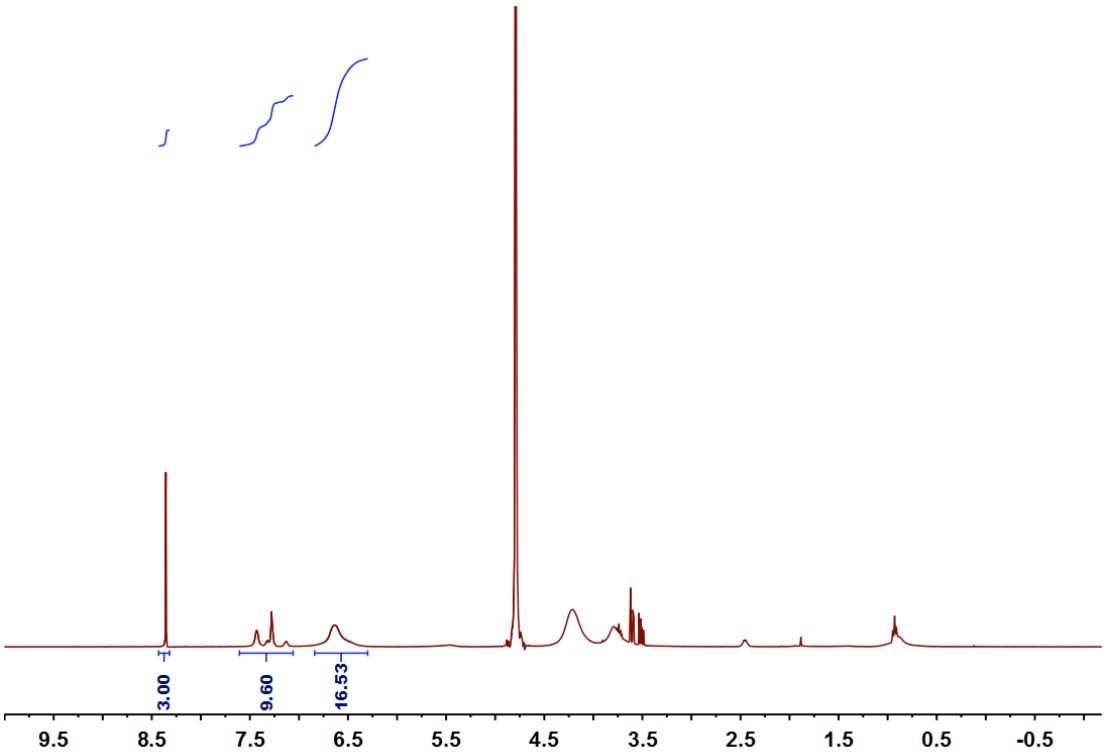


Figure S10. ^{1}H NMR (400 MHz, 298 K, D_2O) spectrum recorded for tamoxifen dissolved in a solution of **WP6** (0.50 mL, 15.0 mM). Here 1.68 mg of sodium benzene-1,3,5-tricarboxylate was added as the reference.

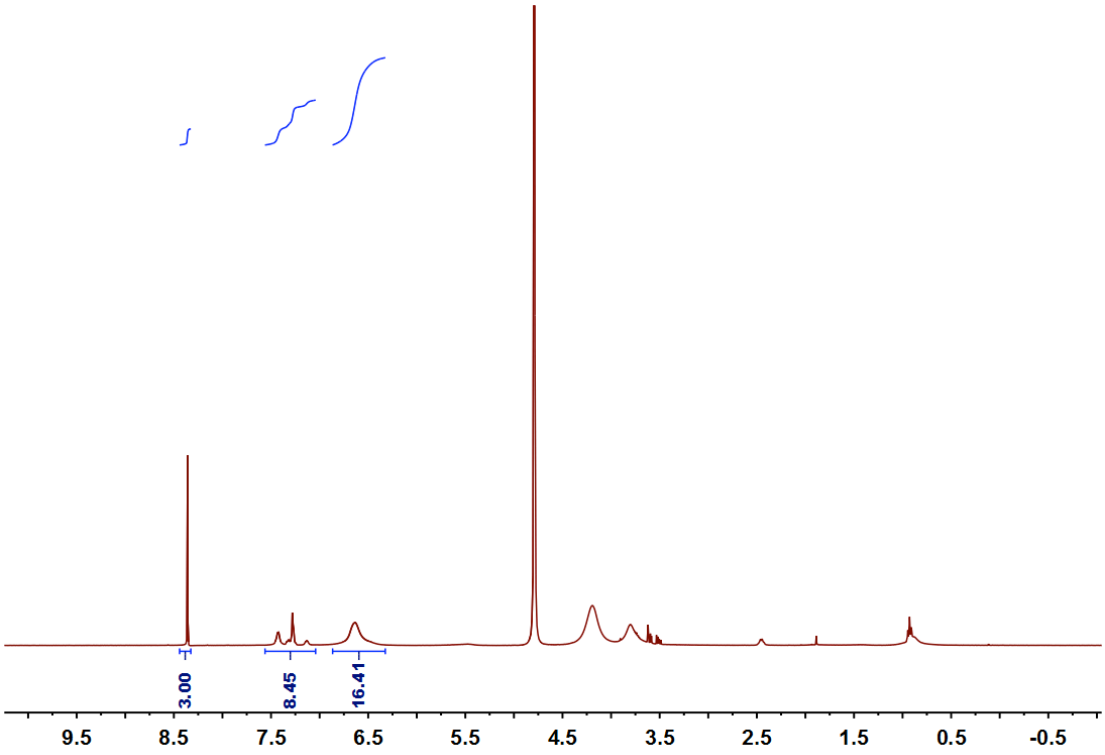


Figure S11. ^{1}H NMR (400 MHz, 298 K, D_2O) spectrum recorded for tamoxifen dissolved in a solution of **WP6** (0.50 mL, 20.0 mM). Here 2.09 mg of sodium benzene-1,3,5-tricarboxylate was added as the reference.

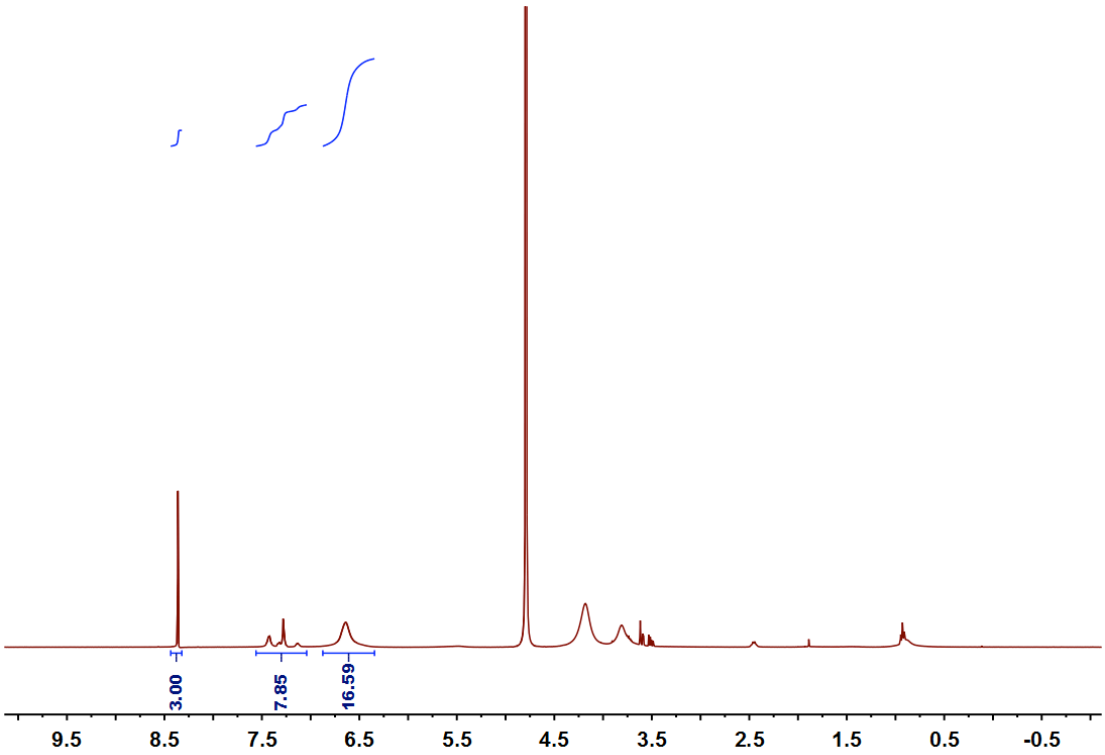


Figure S12. ^{1}H NMR (400 MHz, 298 K, D_2O) spectrum recorded for tamoxifen dissolved in a solution of **WP6** (0.50 mL, 25.0 mM). Here 2.46 mg of sodium benzene-1,3,5-tricarboxylate was added as reference.

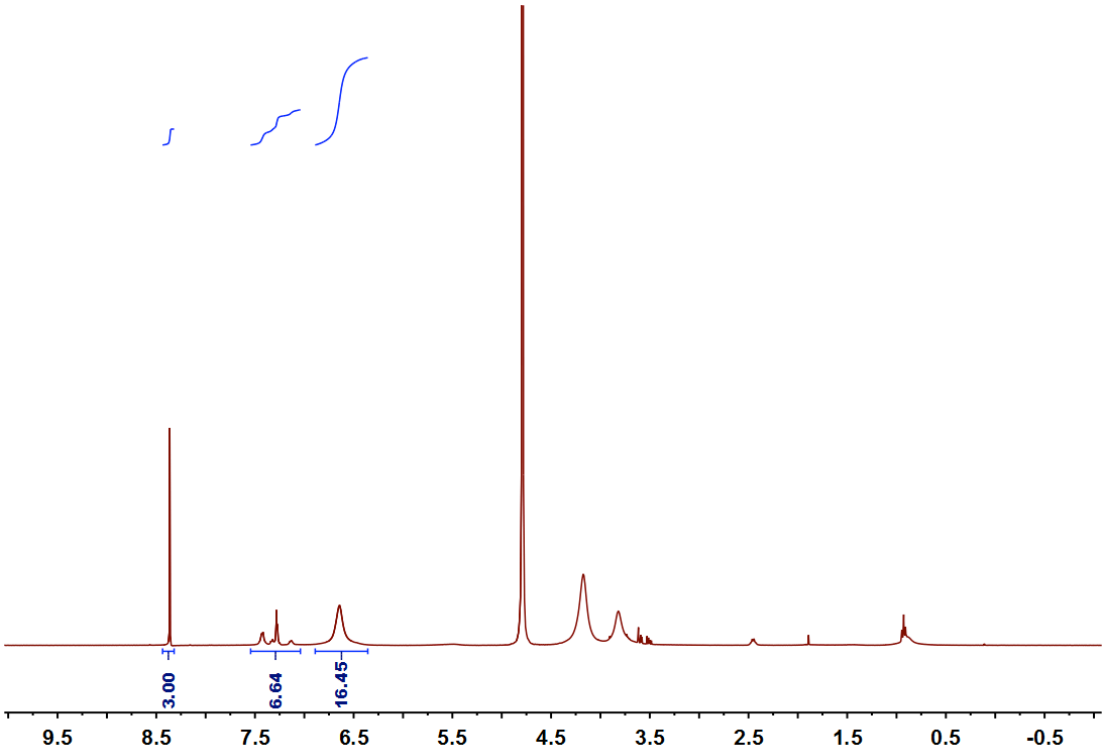


Figure S13. ^{1}H NMR (400 MHz, 298 K, D_2O) spectrum recorded for tamoxifen dissolved in a solution of **WP6** (0.50 mL, 30.0 mM). Here 3.18 mg of sodium benzene-1,3,5-tricarboxylate was added as the reference.

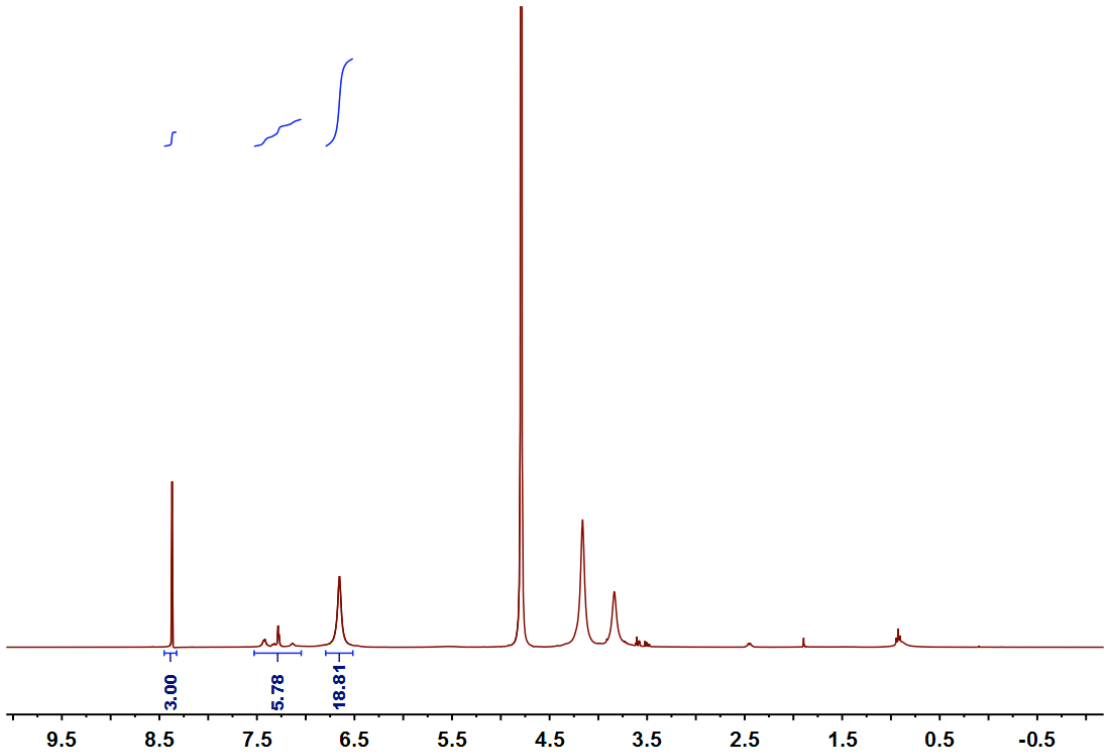


Figure S14. ^{1}H NMR (400 MHz, 298 K, D_2O) spectrum recorded for tamoxifen dissolved in a solution of **WP6** (0.50 mL, 50.0 mM). Here 4.02 mg of sodium benzene-1,3,5-tricarboxylate was added as the reference.

*7. Binding constant determination of **WP6** toward tamoxifen*

The binding constant (K_a) between **WP6** and tamoxifen was calculated based on 1:1 tamoxifen/**WP6** inclusion complex formation according to the method of Higuchi and Connors[S4] using equation S1:

$$K_a = \frac{Slope}{S_0(1 - Slope)} \qquad \text{(eq. S1)}$$

where S_0 is the intrinsic solubility of tamoxifen, $S_0 = 1.60 \times 10^{-5}$ M, and *Slope* is the slope of the linear part of the phase solubility diagram.

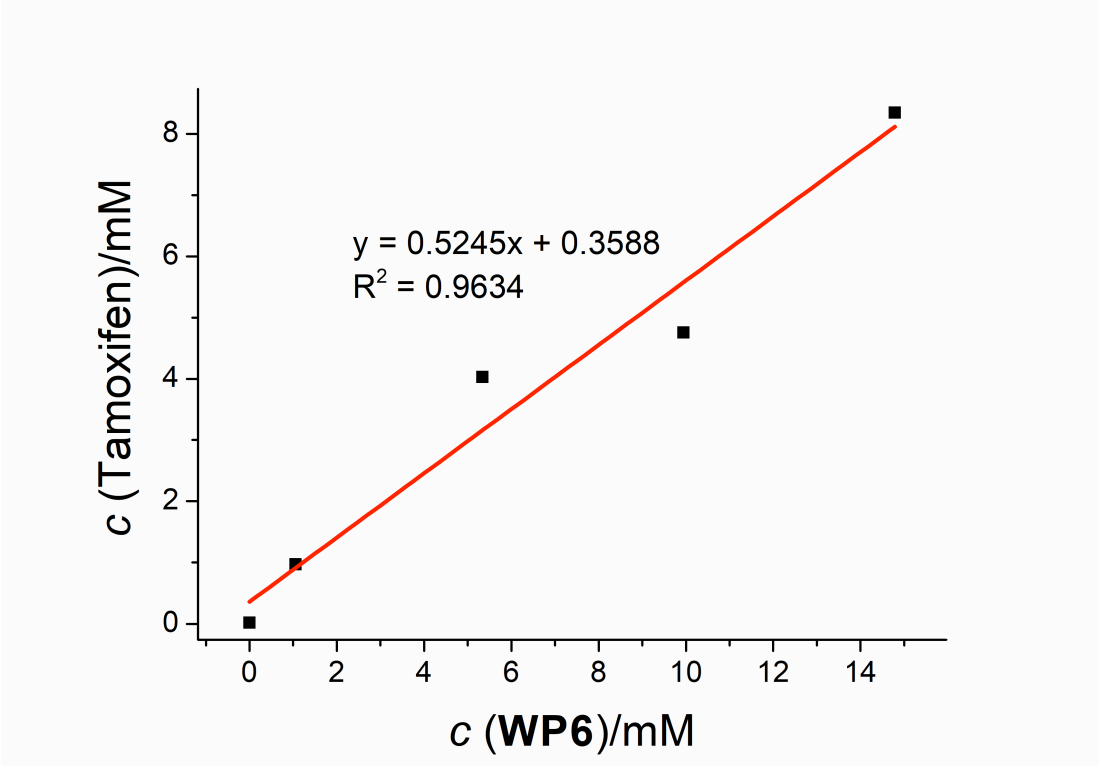


Figure S15. Linear region of the phase solubility diagram for **WP6** and tamoxifen. $K_a = 5.10 \times 10^4$ M^{-1}.

8. Cell viability (MTT) assay after 12 hours of treatment

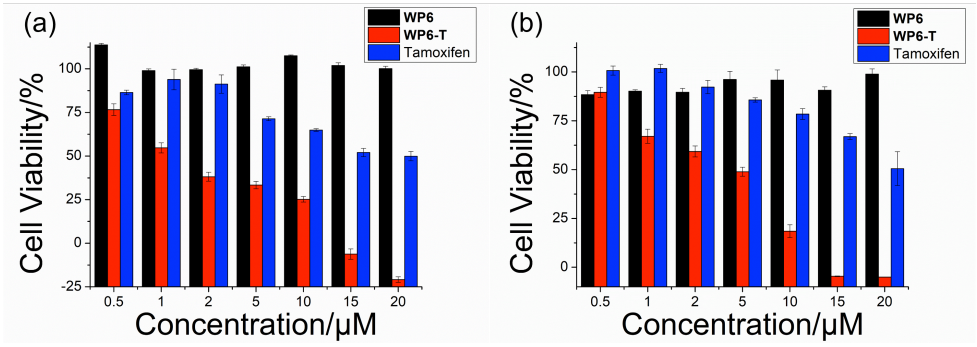


Figure S16. Cell viability of (a) MCF-7 and (b) Hela cells after treatment with different concentrations of **WP6**, **WP6-T** and free drug tamoxifen for 12 hours calculated from MTT assay.

The MTT assay results with 12 hours of incubation are shown in Fig S16. Tamoxifen revealed the killing impact on both the cancer cells after lengthening the incubation time to 12 hours. By contrast, **WP6-T** displayed good killing effect in 4 hours while the free drug tamoxifen did not work. Hence, the solubilizing agent **WP6** can enhance not only the solubility of drug tamoxifen but also the anticancer efficiency of it.

9. References:

S1. D. Xia, G. Yu, J. Li and F. Huang, *Chem. Commun.*, 2014, **50**, 3606.

S2. G. Yu, M. Xue, Z. Zhang, J. Li, C. Han and F. Huang, *J. Am. Chem. Soc.*, 2012, **134**, 13248.

S3. Y. Ma, X. Chi, X. Yan, J. Liu, Y. Yao, W. Chen, F. Huang and J.-L. Hou, *Org. Lett.*, 2012, **14**, 1532.

S4. T. Higuchi and K. A. Connors, *Adv. Anal. Chem. Instrum.*, 1965, **4**, 117.