(Supplementary file)

Thymoquinone chemically conjugated to Doxorubicin: Antitumor activity and subcellular localization

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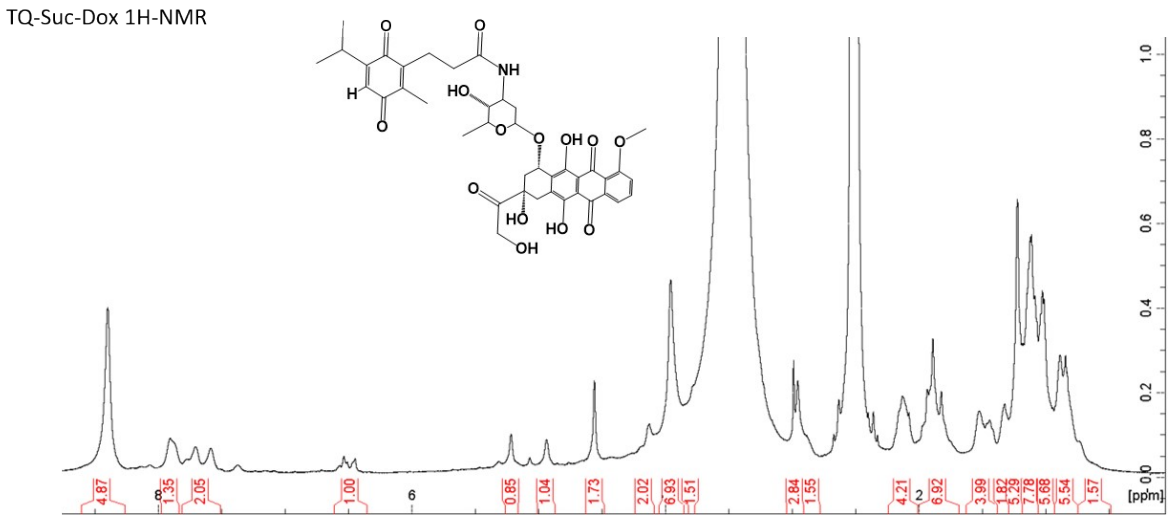
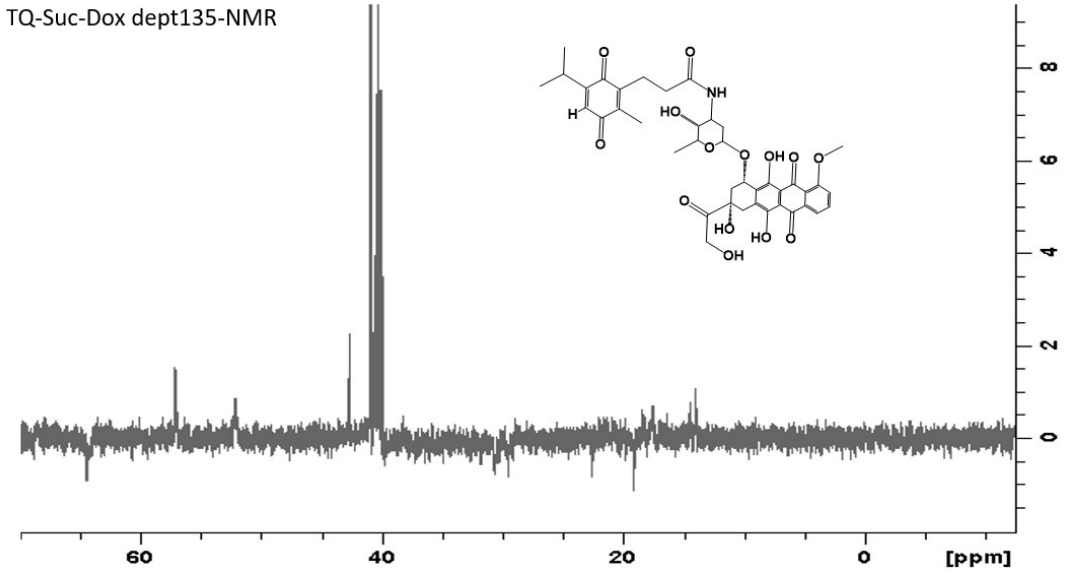
Characterization of TQ-Suc-Dox conjugate

Analysis of both ^{1}H- and ^{13}C-dept135 NMR spectra confirmed the formation of TQ-Suc-Dox. The ^{1}H-NMR data for TQ (structure I) and DOX (structure II) which are the main precursors of TQ-Suc-Dox, are summarized in Tables 1 and 2 to ease comparison with the spectrum of TQ-Suc-Dox. The ^{1}H-NMR spectrum of this compound is shown in Sup fig 1. All protons form the parent compounds appear in the spectrum. The appearance of the proton of the TQ ring is clear at ~6.5 ppm, and several additional signals in the aliphatic region. In addition, upon the formation of TQ-Suc-Dox, several CH_2 can be observed using ^{13}C-dept135-NMR at 19.1, 22.6, 29.4, 30.6, and 64.5 ppm corresponding to 1, 4, 6, b, and the Suc protons (Sup fig 1). However, some of the protons were hard to find due to the solvent signals, so the spectrum was also obtained in CD_3OD. The –CO-*C*H_2-OH protons of CH_2 appeared at 3.5 ppm and were directly connected to the CH_2 carbon at ~70 ppm. The assignments were made based on ^{1}H-^{1}H and ^{1}H-^{13}C correlation NMR experiments. In addition, the amine group (NH_2) in DOX (appears at ~ 7.8 ppm and ~ 42.6 ppm for ^{1}H and ^{15}N atoms respectively) was converted to the amide group (-CO-NH-) which appeared at 7.5 ppm and 150.2 ppm for ^{1}H and ^{15}N atoms respectively (Sup fig 2) which is close to values in the literature.

The formation of TQ-Suc-Dox conjugate was also confirmed using high resolution mass spectrometry. HRMS (ESI) on the negative mode ($[M–H^+]^–$) confirmed TQ-Suc-Dox ($C_{40}H_{43}NO_{14}$) synthesis, where the calculated and the found values were 760.26108 g/mol and 760.26023 g/mol, respectively, with an error of 0.85 mDa (Sup fig 3A). TQ-Suc-Dox synthesis was also confirmed by the positive mode ($[M+Na^+]^+$) with calculated and found values were 784.25758 g/mol and 784.25892 g/mol, respectively, with an error of -1.43 mDa (Sup fig 3B). Also, purity of the generated TQ-Suc-Dox compound was analyzed using HPLC (Shimadzu, Japan), and ~ 90% purity was detected for the compound (Sup fig 4).

Sup. Fig 1





Sup. Fig 1A: ^{1}H-NMR, and ^{13}C-dept-135 NMR spectra for TQ-Suc-Dox in d_6-DMSO at 25 ^{o}C. NMR spectra were obtained using Bruker Biospin AG Magnet system 500MHz/54mm instrument

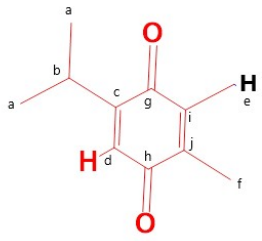


Table 1: ^{1}H-NMR data for TQ Structure I

Type	δ (ppm)	Integration
b	2.96	1, m
a	1.109	6, d
f	1.99	3, d
e	6.5	1, m
d	6.5	1, d

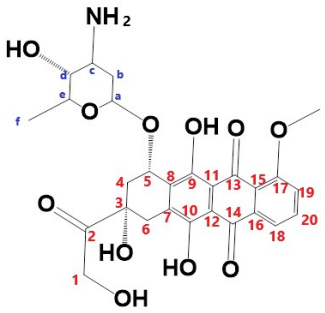


Table 2: ^{1}H-NMR data for Dox in d_6-DMSO. Structure II

proton	δ (ppm)	I	proton	δ (ppm)	I	proton	δ (ppm)	Integration
1	4.54	2, d	18	7.8	1, d**	c	3.3	1, *
3-OH	5.4	1, s ***	19	7.6	1, dd	NH2	7.8	2, **
4	2.08 & 2.12	2, qd	20	7.8	1, t **	d	3.5	1, d
5	4.8	1, t	O-CH3	4.0	3, s	d-OH	5.4	1, s ***
6	2.8 & 2.9	2, dd	1-OH	4.9	1, t	e	4.14	1, m
9-OH	14.0	1, br	a	5.25	1, t	e-CH3	1.12	3, d
10-OH	13.2	1, br	b	1.65 & 1.85	2, dt			

Structure III

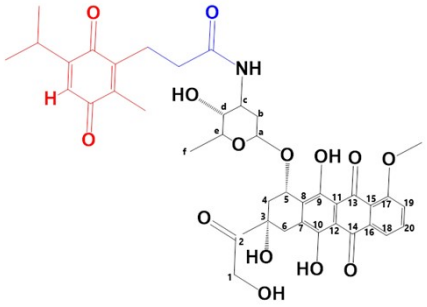
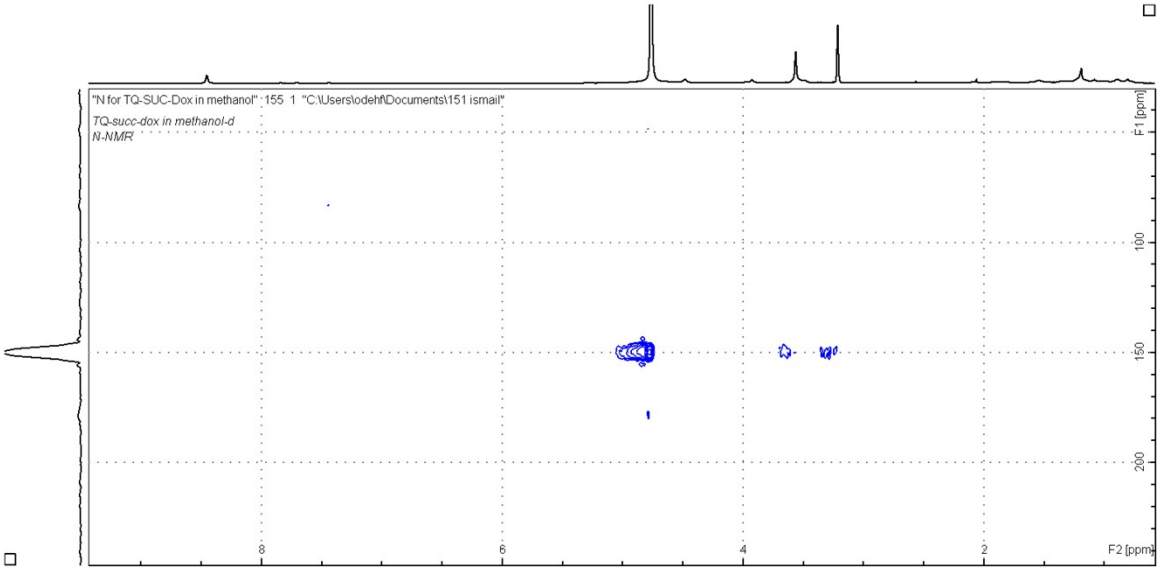


Table 3: ^{1}H-NMR data for TQ-Suc-Dox in d_6-DMSO

proton	δ (ppm)	proton	δ (ppm)	proton	δ (ppm)
1	4.56	18	7.9	d	3.7
1-OH	4.9	19	7.6	e	4.1
3-OH	5.3	20	7.7	f	~1.1
4	2.1	O-CH_3	3.95	CH_{2suc}	3.5
5	4.9	a	5.2	CH_{3TQ}	1.9
9-OH	~14 br	b	1.5	$(CH_3)_2$	~1.1
10-OH	~14 br	c	3.5	CH_{TQ}	2.9

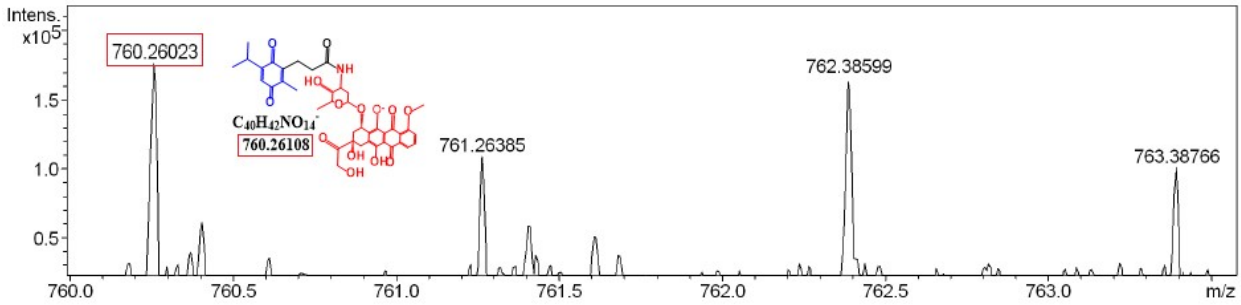
Sup. Fig 2



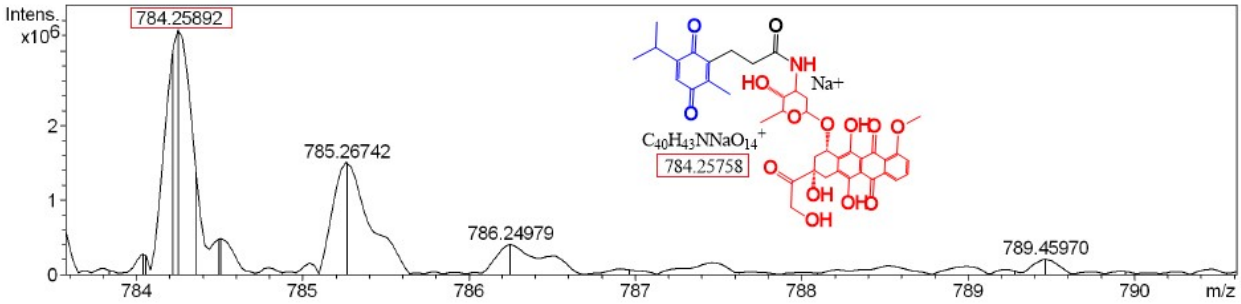
Sup. Fig 2: ^{1}H-^{15}N-HMBC NMR spectrum for TQ-Suc-Dox in CD_3OD

Sup. Fig 3

A)

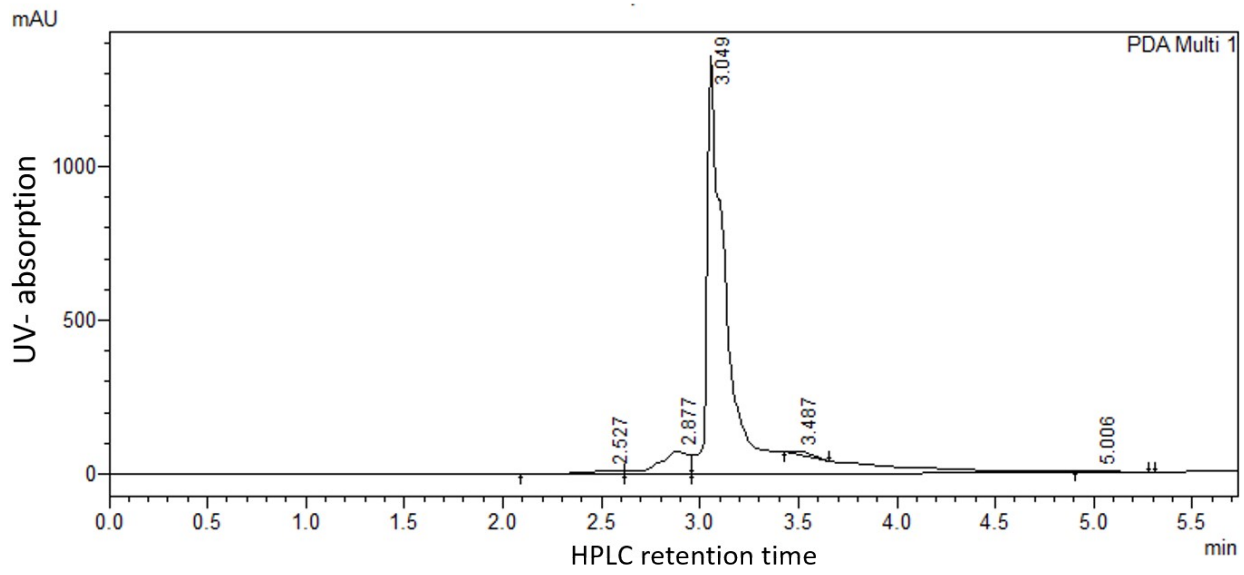


B)



Sup. Fig 3: Mass spectrum of (A) TQ-Suc-Dox on ($[M–H^+]^–$) (B) TQ-Suc-Dox on ($[M+Na^+]^+$). High-resolution mass spectra (HR-MS) used the electrospray ion trap (ESI) technique by collision-induced dissociation on a Bruker APEX-4 (7 Tesla).

Sup. Fig 4



Sup. Fig 4. Reverse phase HPLC analysis, C18 column has been used, the mobile phase composed of methanol: acetonitrile (60:40 volume ratio), the flow rate 1 ml/min and the detection wavelength at 254 nm.