Supplementary Material (ESI) for Organic and Biomolecular Chemistry

Supporting Information

for

pH-Sensitive, *N*-Ethoxybenzylimidazole (NEBI) Bifunctional Crosslinkers Enable Triggered Release of Therapeutics from Drug Delivery Carriers

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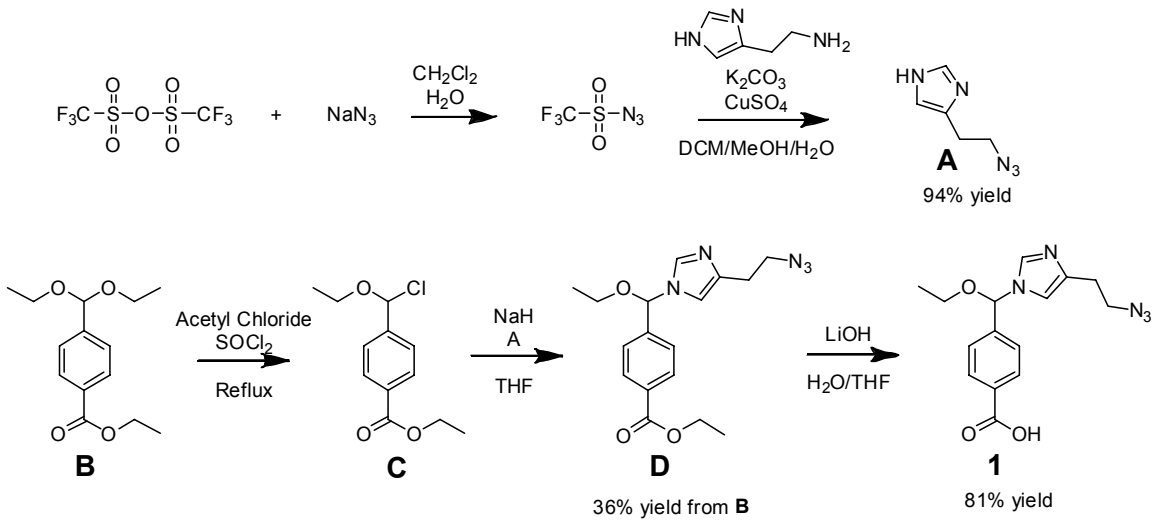
Materials

All reagents were purchased from Sigma-Aldrich, Inc., TCI, or Alfa Aesar and used without further purification. Doxorubicin (aka Adriamycin) was from Bedford Laboratories. NMR spectra were recorded on a Varian 400 (FT, 400 MHz, ^{1}H; 100 MHz, ^{13}C) and Joel ECA-500 (FT, 500MHZ ^{1}H) spectrometer. HR-MS (high-resolution mass spectra) were obtained in the Department of Chemistry & Biochemistry, University of California, San Diego using a ThermoFinnigan MAT900XL-MS. ESI–MS (electrospray ionization mass spectra) were obtained using ThermoFinnigan LCQDECA-MS. Kinetic analysis by reverse-phase high performance liquid chromatography (RP-HPLC) was performed with an Agilent 1100 Series HPLC using an analytical reverse-phase column (SPHERI-5 Phenyl 5 micron, 250 x 4.6 mm). Purification of HSA conjugates were performed with an Atka Purifier 10 using a HiLoad 16/60 superdex 200 prep grade column.

Synthetic Methods

*Scheme for the synthesis of bifunctional NEBI crosslinker (**1**)*



Synthesis of A:

Triflyl azide was prepared following the procedure reported by Pelletier and coworkers.[1] A solution of sodium azide (7.12 g, 110 mmol) was dissolved in distilled H_2O (18 mL) with dichloromethane (DCM, 30 mL) and cooled in an ice bath. Triflyl anhydride (3.7 mL, 20.6 mmol) was added slowly over 20

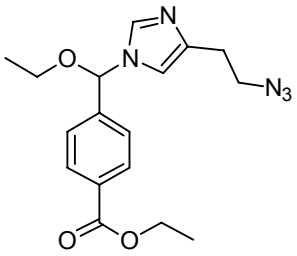
minutes with vigorous stirring continued for an additional two hours. The mixture was placed in a separatory funnel and the organic phase removed. The aqueous portion was extracted with DCM (2 × 30 mL). The organic fractions containing the triflyl azide, were pooled and washed once with saturated Na_2CO_3 and used without further purification. Histamine•2HCl (2.0 g, 11.2 mmol), K_2CO_3 (2.31 g, 16.7 mmol), and $CuSO_4$•pentahydrate (28 mg, 111 μmol) were dissolved in distilled H_2O (15 mL, pH adjusted to 8.3) and methanol (MeOH, 30 mL). The triflyl azide in DCM (90 mL) was added and the mixture was stirred at ambient temperature overnight. The mixture was washed 3 times with ethyl acetate (EtOAc). The organic layers were pooled and dried over Na_2SO_4, and solvent was removed under reduced pressure. Compound **A** was isolated by silica chromatography using as eluent a 96:4:2 mixture of DCM:MeOH:triethylamine. The isolated yield of compound **A** was 94%. Characterization of **A**: ^{1}H NMR ($CDCl_3$, 400 MHz) 2.904 (t, 2H), 3.587 (t, 2H), 6.899 (s, 1H), 7.630 (s, 1H) ESI-MS (*m/z*, $M+H^+$): 137.99

Synthesis of D

The synthesis of **B** was described in previous work by Kong et al.[2] Compound **B** (555 mg, 2.2 mmol), freshly distilled acetyl chloride (250 μL, 3.52 mmol) and thionyl chloride (52 μL, 440 μmol) were combined without additional solvent and refluxed for 1 hour at 65ºC under N_2. The excess thionyl chloride and acetyl chloride were removed under reduced pressure and the crude mixture was characterized by ^{1}H NMR. ^{1}H-NMR (in $CDCl_3$) indicated a new peak at 6.203 ppm, presumably corresponding to the benzylic H in **C**. The crude yield of **C** by ^{1}H-NMR was ~54%, with the remainder of the material identified as starting material **B** (~8%) as well as a large amount (~38%) of ethyl 4-formylbenzoate. The crude material was immediately taken on to the next step without further purification.

In a separate dry flask, n-BuLi (1.43mL of a 2 M solution in hexane) was added to a solution of **A** (300 mg, 2.2 mmol) dissolved in dry tetrahydrofuran (THF). The solution was allowed to stir for 1 hour at 23ºC, and then the crude mixture of **C** was added dropwise to the **A**/n-BuLi solution. The combined

solution was stirred for 12 hours at 23ºC. Saturated Na_2CO_3 was added to the solution and washed 3 times with DCM. The organic layers were pooled and dried over Na_2SO_4. The solvent was removed under reduced pressure. Compound **D** was isolated by silica chromatography using as eluent a 97:2:1 mixture of DCM:MeOH:triethylamine. This procedure produced a ~ 1:1 mixture of isomers (with respect to the nitrogens on the imidazole moiety) in 36% isolated yield over two steps. Although the desired isomers could be resolved and purified by chromatography for characterization by ^{1}H NMR, ^{13}C NMR and HR-MS, we used this mixture of isomers for the synthesis of **3** since using this mixture significantly simplified the synthesis and purification of the conjugates and since separating these isomers was not expected to affect the results of the described fluorescence imaging and cytotoxicity studies.



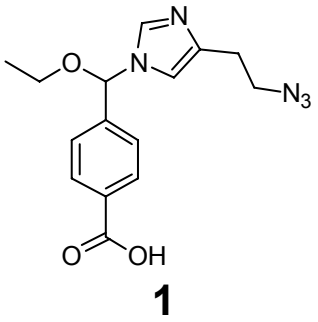
D

Characterization of **D**: ^{1}H NMR ($CDCl_3$, 400 MHz) 1.296 (t, 3H), 1.390 (t, 3H), 2.849 (t, 2H), 3.570 (m, 4H), 4.388 (q, 2H), 6.198 (s, 1H), 6.773 (s, 1H), 7.426 (d, 2H), 7.758 (s, 1H), 8.056 (d, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz) 14.268, 14.708, 28.415, 50.647, 61.146, 64.635, 86.383, 114.358, 125.942, 129.824, 131.118, 136.295, 139.944, 142.295, 165.977. HR-ESI-FT-MS calcd *(m/z)*: 344.1717; found: 344.1721. Inspection by NOESY indicated a correlated peak between the benzylic hydrogen and the hydrogen on C-5 of the imidazole moiety, confirming the probable identity of isomer **D** with respect to the two nitrogens on the imidazole group.

Synthesis of compound 1

Compound **D** (6.6 mg, 9.7 μmol) was dissolved in 200 μL of THF and 100 μL of distilled H_2O. A solution of LiOH (100 μL of a 1M solution) was added and allowed to stir for 12 hours at at 4ºC. Saturated NH_4Cl was added to the solution and it was washed 3 times with EtOAc. The organic layers

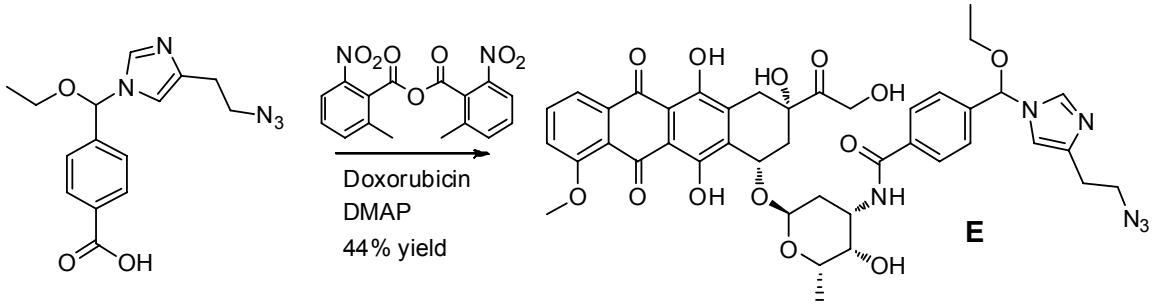
were pooled and dried with Na_2SO_4. The solvent was removed under reduced pressure to give **1** in 81% isolated yield.



1

Characterization of **1:** ^{1}H NMR (CD_3OD, 400 MHz) 1.230 (t, 3H), 2.762 (t, 2H), 3.271 (t, 2H), 3.447 (t, 2H), 6.374 (s, 1H), 6.937 (s, 1H) , 7.331 (d, 2H), 7.825 (s, 1H), 7.893 (d, 2H) ^{13}C NMR (CD_3OD, 100 MHz) 15.400, 29.242, 52.066, 65.785, 88.265, 116.293, 126.698, 130.701, 138.113, 140.033, 140.452, 141.687, 174.975. HR-ESI-FT-MS (*m/z*) calcd: 316.1404; found: 316.1410.

Synthesis of doxorubicn-NEBI-azide (E)

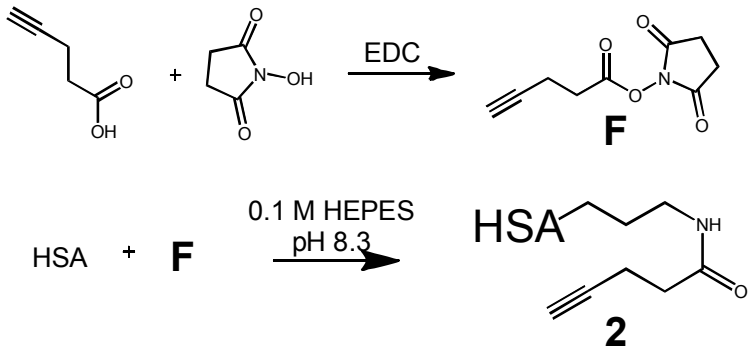


E

A solution of **1** (3.0 mg, 9.5 μmol), 2-Methyl-6-nitrobenzoic anhydride (2.9 mg, 8.6 μmol), Dimethylaminopyridine (DMAP, 1 crystal) in dimethylformamide (DMF) was stirred for 2 hours at 23ºC. Doxorubicin•HCl (5 mg, 8.6 μmol) dissolved in 1mL DMF was added to the solution and allowed to stir for an additional 12 hours. The DMF was removed under reduced pressure. Doxorubicin-NEBI-azide **E** was isolated by silica column chromatography using as eluent 96:3:1 mixture of DCM:MeOH:triethylamine. The isolated yield of E was 44%. Characterization of **E:** ^{1}H NMR ($CDCl_3$, 500 MHz) 1.270 (t, 3H), 1.305/1.318 (d, 3H), 1.968 (s, 1H), 2.014 (s, 1H), 2.171 (d, 1H), 2.179 (d, 1H), 2.814 (t, 2H), 3.309 (m, 2H), 3.526 (t, 2H), 3.566 (m, 2H), 3.746 (s, 1H), 4.078 (s, 3H), 4.221 (m, 1H),

4.369 (t, 1H), 4.781 (s, 2 H), 5.321 (s, 1H), 5.549 (s, 1H), 6.131 (s, 1H), 6.730 (s, 1H), 7.359 (m, 3H), 7.622 (s, 1H), 7.726 (d, 2H), 7.794 (t, 1H), 8.042 (d, 1H). HR-MS (*m/z*) calcd: 841.3039; found: 841.3054.

*Scheme for the synthesis of compound **2***



Synthesis of F

A solution of Pent-4-ynoic acid (10.1 mmol, 1 g), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 12.2 mmol, 2.34 g), *N*-Hydroxysuccinimide (12.2 mmol, 1.41 g) was stirred at room temperature in 100 mL of dry DCM for 24 hours. Saturated Na_2CO_3 was added to the solution, and washed 3 times with DCM. The organic layers were pooled, dried over Na_2SO_4 and solvent removed under reduced pressure to give a 76% crude yield of compound **F**. The crude sample was used in subsequent reaction with HSA without further purification. Characterization of **F**: ^{1}H NMR ($CDCl_3$, 400 MHz) 2.049 (t, 1H), 2.621 (m, 2H), 2.848-2.903 (m, 6H).

Synthesis of HSA-alkyne 2

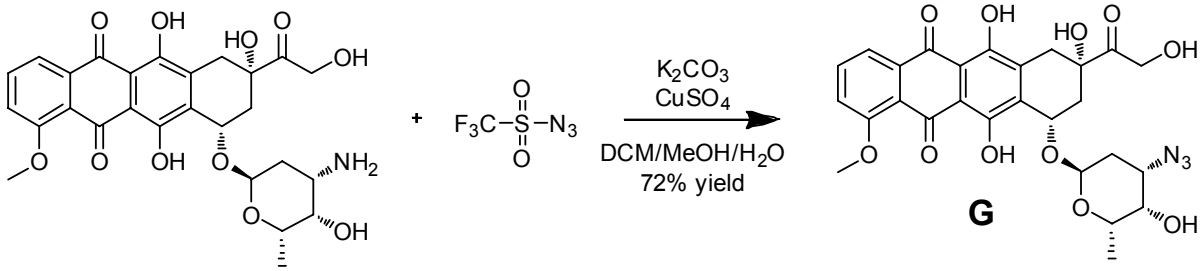
Human serum albumin (HSA, 200 mg, 3 μmol) was dissolved in 12 mL of 0.1 M HEPES buffer, pH 8.3. Compound **F** (300 μmol) was dissolved in 1 mL of dimethyl sulfoxide (DMSO) and added to the HSA solution dropwise at 23ºC. The reaction was monitored on a pH meter. Aliquots of 0.1 M NaOH were used to maintain a pH of 8.3 over the course of the reaction of HSA and **F**. After complete addition of **F**, the reaction was allowed to stir for an additional 1 hour at 23ºC. Compound **2** was exchanged into

phosphate buffered saline (PBS, pH 8.0) using Amicon ultra centrifugal filter devices (30kD MW cut off, catalog number: UFC903024).

Synthesis of HSA-NEBI-doxorubicin (3)

The click reaction was performed following the general procedure previously reported by Speers, et. al.[3] A solution containing 43 mL of a 1.5 mg/mL solution of HSA-alkyne (**2**) in PBS (pH 8.0) was combined with 1 mL of a 2.5 mM solution of doxorubicin-NEBI-azide **E** in DMSO at 23ºC. The solution was vortexed for 5 seconds. A solution of freshly made tris(2-carboxyethyl)phosphine (TCEP, 1 mL, 50 mM) and tris-(benzyltriazolylmethyl)amine (TBTA) in 1:4 DMSO:t-BuOH (3.0 mL, 1.7 mM) were added to the solution containing **2** and **E** and vortexed for 5 seconds. A solution of $CuSO_4$ in water (1 mL, 50 mM) was added and the solution was vortex again for 5 seconds. The reaction was stirred for 1 hour at ambient temperature. The solution was exchanged into PBS pH 7.4 using Amicon Ultra Centrifugal Filter devices (30kD MW cut off). Compound **3** was purified by FPLC using a superdex 200 prep grade column with PBS at pH 7.4 with a flow rate of 1 mL min^{-1}. The retention time was 40-50 minutes. The characterization of **3** is described later in this supporting information after the description of the synthesis of compound **4**.

Synthesis of Azido-doxorubicin (G)



Triflyl azide was prepared following the procedure reported by Pelletier and coworkers.[1] A solution of sodium azide (1.78 g, 27.5 mmol) was dissolved in distilled H_2O (4.5 mL) with DCM (7.5 mL) and cooled in an ice bath. Triflyl anhydride (0.9 mL, 5.55 mmol) was added slowly over 5 minutes with

stirring continued for an additional two hours at 23ºC. The mixture was placed in a separatory funnel and the organic phase removed. The aqueous portion was extracted with DCM (2 × 3.75 mL). The organic fractions containing the triflyl azide, were pooled and washed once with saturated Na_2CO_3 and used without further purification. The total volume of trifyl azide in DCM was ~15 mL. Triflyl azide (150 μL in DCM solution) was added to a solution containing doxorubicin•HCl (8.62 μmol), $CuSO_4$ (86 nmol), and K_2CO_3(12.9 μmol) in a 1:1:1 mixture of DCM:H_2O:MeOH. The solution was stirred for 12 hours at ambient temperature. The solvent was removed under reduced pressure and azido-doxorubicin **G** was purified over silica using as eluent 97:3 DCM:MeOH. The yield of **G** was 20%.

Compound **G** was also evaluated for cytotoxic activity against human ovarian carcinoma 2008 cells using a standard SRB assay as described in the main text. These studies revealed that compound **G** reduced the viability of the cells with an IC_{50} of 80 nM.

Characterization of **G**: ^{1}H NMR ($CDCl_3$, 500 MHz) 1.343 (d, 3H), 1.928 (m, 1H), 2.109 (m, 1H), 2.202 (m, 1H), 2.331 (d, 1H), 3.078 (d, 1H), 3.286 (d,d 1H), 3.591 (m, 1H), 3.735 (s, 1H), 4.035 (d, 1H), 4.098 (s, 3H), 4.753 (s, 2H), 5.330 (m, 1H), 5.593 (d, 1H), 7.419 (d, 1H), 7.802 (t, 1H), 8.042 (d, 1H). HR-ESI-FT-MS: calcd (*m/z,* $M+Na^+$): 592.1547; found: 592.1538.

Synthesis of HSA-doxorubicin conjugate (4)

The click reaction was performed following the general procedure previously reported by Speers, et. al.[3] A solution containing 43 mL of a 1.5 mg/mL solution of HSA-alkyne (**2**) in PBS (pH 8.0) was combined with 1 mL of a 2.5 mM solution of doxorubicin-azide **G** in DMSO. The solution was vortexed for 5 seconds. A solution of freshly made TCEP (1 mL, 50 mM) and TBTA in 1:4 DMSO:t-BuOH (3.0 mL, 1.7 mM) were added to the solution containing **2** and **G** and vortexed for 5 seconds. A solution of $CuSO_4$ in water (1 mL, 50 mM) was added and the solution was vortex again for 5 seconds. The reaction was stirred for 1 hour at ambient temperature. The solution was exchanged into PBS pH 7.4 using Amicon Ultra Centrifugal Filter devices (30kD MW cutoff). Compound **4** was purified by FPLC using a superdex

200 prep grade column with PBS at pH 7.4 with a flow rate of 1 mL min^{-1}; the retention time was 40-50 minutes.

Characterization of HSA-NEBI-doxorubicin (3) and HSA-doxorubicin (4).

The Bradford assay[4] was used to determine the concentration of HSA. The extinction coefficient (ε) of doxorubicin in PBS pH 7.4 at λ = 480 nm was measured (3.37 mM^{-1}), and Beer-Lambert Law[4] was used to determine the concentration of doxorubicin in the sample. This analysis afforded an estimate of ~1 doxorubicin molecule per HSA molecule in compound **3**. Similarly, this procedure afforded an estimate of ~1 doxorubicin molecule per HSA molecule in compound **4**.

Compounds **3** and **4** were also characterized by 4-20% SDS PAGE gel (Figure **S1**). The gel was analyzed for fluorescence using a Typhoon imager (Ex: 488nm, Em/bp: 580/30nm), followed by staining with Coomassie blue. Figure S1 shows an image of the SDS-PAGE gel by fluorescence analysis and by Coomassie blue staining for HSA conjugates **2-4** and for crude samples from control reactions.

Lanes 5 and 6 contained a mixture of doxorubicin-NEBI-azide **E** with HSA-alkyne **2** or doxorubicin-azide **G** with **2** that were reacted in the same manner as for the synthesis of **3** and **4** but without the addition of the copper catalyst. The absence of fluorescence on the HSA resulting from these control reactions strongly supports that doxorubicin is covalently conjugated to HSA in **3** and **4**, presumably through the designed "click" reactions. Importantly, these control reactions demonstrate that the conditions used to synthesize and to purify **3** and **4** do not result in non-specific association of doxorubicin derivatives **E** and **G** with HSA.

SDS-PAGE Gel	Coomassie Blue Stain							Fluorescent Image					
Lane	1	2	3	4	5	6		1	2	3	4	5	6
HSA (66 kDa)	+	-	-	-	-	-		+	-	-	-	-	-
HSA-alkyne (**2**)	-	+	+	+	+	+		-	+	+	+	+	+
Doxorubcin azide (**G**)	-	-	+	-	+	-		-	-	+	-	+	-
Dox-NEBI-azide (**E**)	-	-	-	+	-	+		-	-	-	+	-	+
TCEP	-	-	+	+	+	+		-	-	+	+	+	+
$CuSO_4$	-	-	+	+	-	-		-	-	+	+	-	-

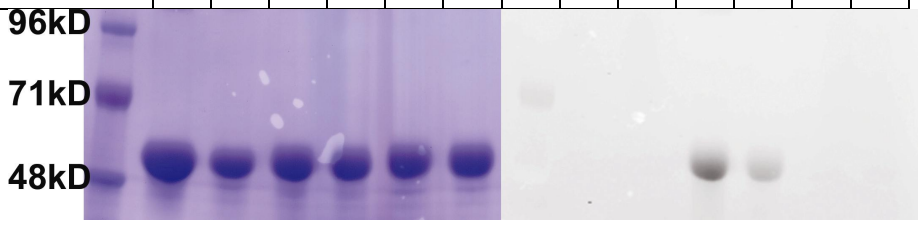
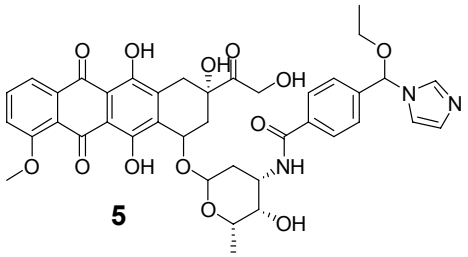


Figure S1. SDS-PAGE gel characterization of compounds **2-4** and crude mixture from control reactions. Compound **2** is in lane 2, compound **3** is in lane 4, and compound **4** is in lane 3. The gel was analyzed by Coomassie Blue staining (left) and fluorescence imaging (right). The legend indicates the components used to generate the protein species in each lane of the gel.

Synthesis of 5



5

The synthesis of **5** was previously reported by Kong et al.[2] Characterization of **5**: ^{1}H NMR (CD_3OD, 400 MHz) 1.242 (t, 3H), 1.283 (m, 6H), 1.833 (d, 1H), 2.136 (m, 2H), 2.356 (d, 1H), 2.915 (d,1H), 3.017 (d, 1H), 3.491 (m, 1H), 3.635 (m, 1H), 3.741 (s, 1H), 3.955 (s, 3H), 4.326 (t, 2H), 4.753 (d, 2 H), 5.069 (s, 1H), 5.435 (s, 1H), 5.492 (s, 1H), 6.471 (s, 1H), 6.990(s, 1H), 7.081 (s, 1H), 7.433 (m, 3 H), 7.740 (t, 1H), 7.809 (d, 3H), 7.885 (s, 1H). HR-MS (*m/z*) calcd ($M+H^+$): 772.2712; found, 772.2721.

Fluorescence Cellular Imaging Studies

Human ovarian carcinoma 2008 cells were plated with Roswell Park Memorial Institute-1640 (RMPI-1640, Invitrogen 11835-030) phenol red free media supplemented with 10% FBS (Omega Scientific, FB-01) on 35 mm glass bottom dishes (MatTek Co., Ashland, MA) and incubated overnight. HSA-NEBI-doxorubicin (**3**) was added to the cells to give a final concentration of 10 μM of **3** and allowed to incubate for 24 hours. Lysotracker Blue (Invitrogen, L7525) was added to the cells and incubated for 30 minutes. The remaining living cells were washed three times with phenol red free RPMI-1640 media without FBS and then immediately imaged with a Delta Vision Deconvolution Microscope System (Applied Precision, Issaquah, WA) equipped with a Nikon TE-200 inverted light microscope with infinity corrected lenses and with a mercury arc lamp as the illumination source. The co-localization was determined using softWoRx image analysis software (Applied Precision, Issaquah, WA).

The intrinsic fluorescence of doxorubicin (Abs/Em: 480/550, 585 nm) was used to visualize the location of **3** and was observed by excitation with bandpass (bp) filtered light (Ex/bp: 490/20nm) and the emission monitored at Em/bp: 617/73 nm. The fluorescence of the Lysotracer Blue (Abs/Em: 373/422 nm) was detected using an Ex/bp: 360/40 nm excitation filter and an Em/bp: 457/50 nm emission filter.

In order to provide a quantifiable value for the co-localization of Lysotracker Blue and **3**, we determined the extent of overlap of the fluorescence of Lysotracker Blue and **3.** This imaging analysis afforded a Pearson's correlation coefficient[5] of 0.71.

Cytotoxicity Studies

Human ovarian carcinoma 2008 cells were plated into each well of a 96 well plate (3000 cells/well) using RPMI-1640 media with 10% FBS. The cells were incubated for 24 hours at 37˚C with 5% CO_2. After the incubation period, cells were dosed with various concentrations of HSA, **2-4**, **6**, or 4-carboxybenzaldehyde. The cells were allowed to incubate for 72 hours. After incubation, cells were washed 3 times with 200 μL of PBS (Mediatech, 46-013CM) pH 7.4. Cells were then fixed with 200 μL of PBS and 50 μL of 50% trichloroacetic acid for one hour at 4°C. After fixation, cells were washed 5

times with water and allowed to dry. A 0.4% sulforhodamine B (SRB, Sigma Aldrich, S1402) solution in 1% acetic acid was added to the cells and incubated for 15 minutes at room temperature. The cells were washed 3 times with 1% acetic acid and the 96 well plate was allowed to dry. Tris base solution (10 mM, 200 μL) was added to the wells for 15 minutes prior to measuring the absorbance at 515 nm. All data points represent the UV absorbance of SRB relative to a sample of cells that were fixed at time zero of the incubation period (i.e., 24 hours after introduction of cells to the wells of the 96 well plate). Reference 37 reports a similar protocol for estimating cell viability.

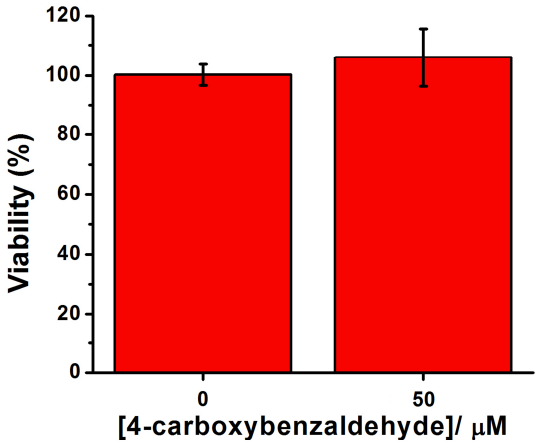


Figure S2. Comparison of the viability of human ovarian carcinoma 2008 cells with or without exposure to a 50 μM concentration of 4-carboxybenzaldehyde. The viability of cells exposed to 4-carboxybenzladehyde were not significantly different than the viability of untreated control cells.

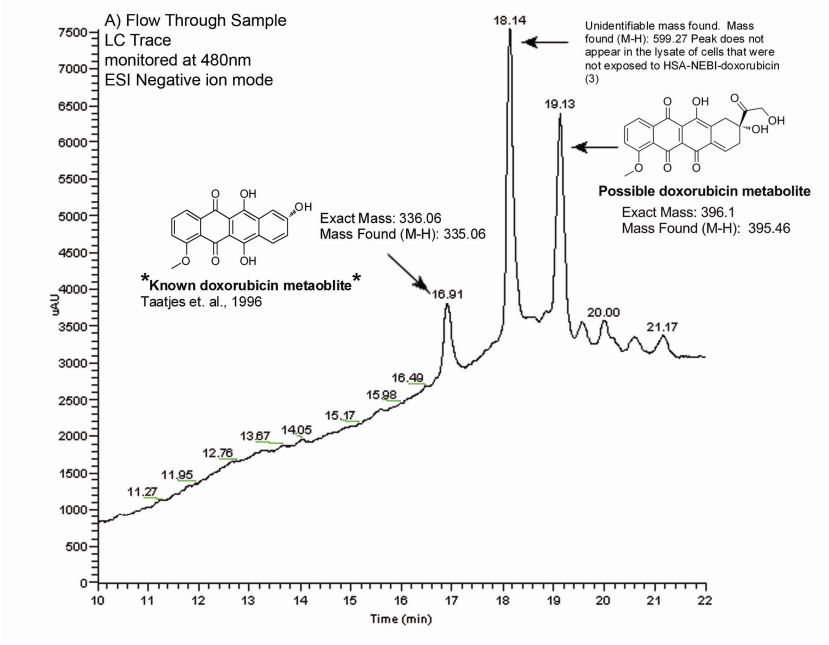
HSA-NEBI-doxorubicin (3) Cell Uptake Studies

Human ovarian carcinoma 2008 cells were plated with RMPI-1640, phenol red free media supplemented with 10% FBS on 35 mm glass bottom dishes and incubated overnight. HSA-NEBI-doxorubicin (**3**) and doxorubicin analog (**6**) were incubated with cells for 3 hours. The final concentration of doxorubicin in the samples were 20 μM. The cells were washed three times with media to remove excess molecules and then immediately imaged with a Delta Vision Deconvolution Microscope System (Applied Precision, Issaquah, WA) equipped with a Nikon TE-200 inverted light microscope with infinity corrected lenses and with a mercury arc lamp as the illumination source. Doxorubicin was observed by

excitation with bandpass (bp) filtered light (Ex/bp: 490/20nm) and the emission monitored at Em/bp: 457/50 nm. The average fluorescence intensity per cell was determined using softWoRx image analysis software (Applied Precision, Issaquah, WA).

LC-MS analysis of the breakdown of HSA-NEBI-doxorubicin (3) in cells

Human ovarian carcinoma 2008 cells were plated into 60mm x 15 mm tissue culture treated dishes with RPMI-1640 phenol red free media with 10% FBS. The cells were incubated at 37˚C with 5% CO_2 until ~ 80% confluent. HSA-NEBI-doxorubicin **(3)** was added to the cells and allowed to incubate for 3 days. After 3 days, the cells were lysed with 0.1% triton X in PBS solution. The media and PBS was combined and run through a Supelclean™ Hisep™ SPE Tube. The flow through was collected. Additionally, we subsequently eluted the Hisep SPE Tube with methanol. LC-MS was used to analyze both the flow through and methanol samples. The LC was monitored at 480nm, the absorbance of doxorubicin, to highlight the chromatographic peaks that possibly contained doxorubicin-derived species[6, 7]. The method used for elution was a gradient from 30% methanol and 70% water to 70% methanol and 30% water over 20 minutes with a flow rate of 1 ml min^{-1} on a reverse phase column (Phenomenex Synergi Polar-RP LC column 4 micron, 150mm x 4.6mm). Negative ion mode The mass spectrometry data was obtained using ThermoFinnigan LCQDECA-MS in ESI-Negative ion mode.



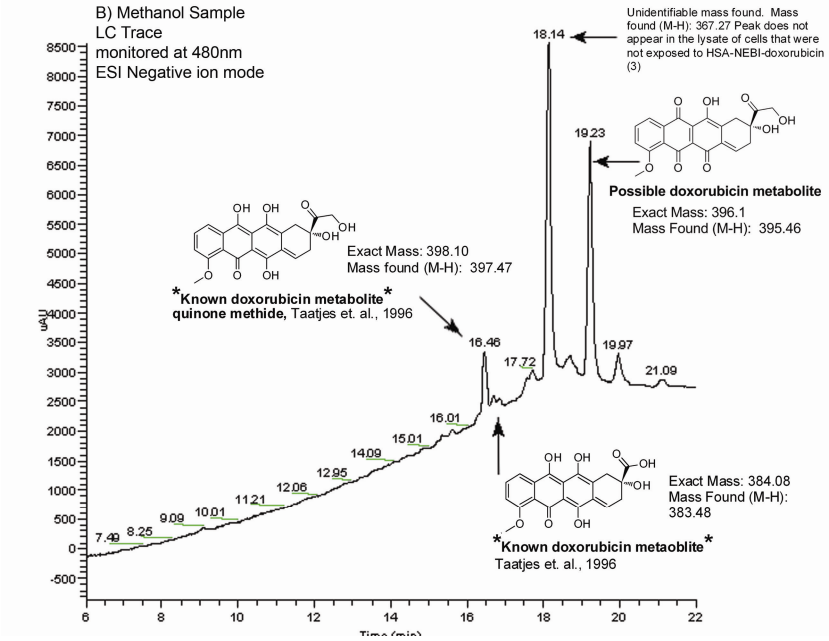


Figure S3 LC-MS analysis of the lysate of human ovarian carcinoma 2008 cells treated with HSA-NEBI-doxorubicin (**3**) and subjected to a Superclean™ HiSep™ SPE tube. A) The LC trace of the flow through sample. B) The LC trace of the methanol sample. The samples were monitored at 480nm (the absorbance of doxorubicin) to highlight possible doxorubicin metabolites. ESI-Negative ion mode was used to determine the mass of the peaks observed at 480 nm.

Measurement of the rate of hydrolysis of N-ethoxybenzylimidazole 7 in the presence of Metal Lewis Acids or Serum.

Solutions containing 0.5 mM *N*-ethoxybenzylimidazoles **7** were incubated in 0.1 M MES buffer (pH 5.5) or 0.1 M HEPES (pH 7.4) buffer and incubated at 37°C. The hydrolysis of **7** in the presence of various metal Lewis acids was monitored by observing the formation of benzaldehyde product by UV absorbance at 235 nm. Solutions of metal ions were made from the following salts: $Zn(NO_3)_2$, $Cu(NO_3)_2$, $MgCl_2$, $FeCl_3$, $CaCl_2$, NaCl, and KCl. For measurement of the hydrolysis of **7** in the presence of fetal bovine serum (FBS), FBS was dialyzed against the solution of 0.5 mM **7** (in 0.1 M PBS buffer, pH 7.4) during the course of the hydrolysis experiment. The dialysis chamber was removed only during measurement of the formation of benzaldehyde product by UV spectroscopy; the dialysis chamber was necessary for these studies since the UV absortion of serum proteins in FBS interfered with the analysis of the hydrolysis of **7**.

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