Electronic Supporting Information

Beta-Cyclodextrin Formulation of a Disulfide-Bond Disrupting Agent for Improved Systemic Exposure

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Method to determine host cavity volume

The method described below was inspired by the work of Grabicki and co-workers and combines the use of Hyperchem 8.0.6 and SwissPDB 4.1.0.[1,2] Both programs are available free of charge from the internet.

Hyperchem: www.hypercubeusa.com/Products/tabid/354/Default.aspx

SwissPDB: https://spdbv.unil.ch

Initial ACD, BCD, and GCD structures were retrieved from the Cambridge Crystallographic Data Center where they are deposited as BAJJAX (1105430), WEWTOJ (762697), and CYDXPL (1134600) respectively using ConQuest 2.0.4. The structures were exported to Mercury 4.3.0 where they were edited and saved as mol files. Mol files were imported into Hyperchem where the structures were geometry optimized at the PM3 level. Optimized structures were saved as mol files.

2D structures of coronene and cicumcoronene, drawn using ChemDraw Professional 19.0 and saved as cdx files, were imported into Chem3D 19.0 where they were saved as mol files. Mol files were imported into Hyperchem where the structures were geometry optimized at the PM3 level. Optimized structures were saved as mol files.

To estimate the cavity volume of BCD

a. In Hyperchem, the structures of BCD and two coronenes are opened in the same window using the "Merge…" feature which allows multiple structures to be accessed simultaneously. (File > Open… then File > Merge… for every additional structure).

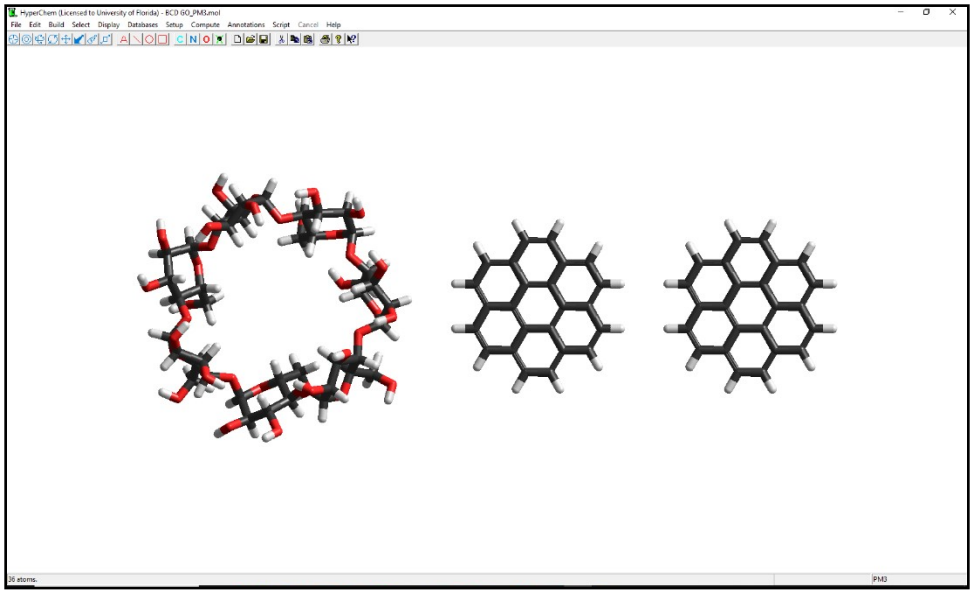


Fig. S1 Minimized structures of BCD (left) and two coronenes (middle and right) merged in Hyperchem.

b. BCD is selected and moved to the Origin (Edit > Translate… > Translate Selection > Origin). In this example, BCD was already at the Origin and remained stationary.

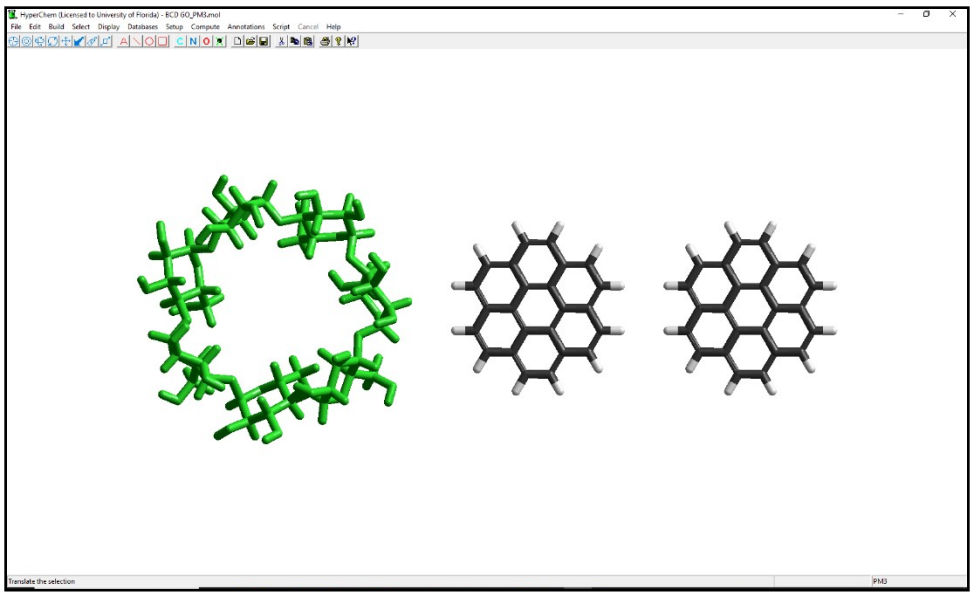


Fig. S2 Selected structure of BCD positioned at the Origin.

c. The same step is repeated with one of the coronenes to position it at the Origin (Edit > Translate… > Translate Selection > Origin). Once at the Origin, the coronene is moved closer to the narrow rim of BCD (Edit > Translate… > Translate Selection > Other [X = 0.000, Y = 0.000, Z = -1.600]).

d. The steps in c are repeated with the second coronene to position it closer to the wide rim of BCD (Edit > Translate… > Translate Selection > Origin then Edit > Translate… > Translate Selection > Other [X = 0.000, Y = 0.000, Z = +1.900]).

e. At this point, a “capped” BCD is obtained which is saved as a mol file. Note that the orientation of the viewer is changed in Fig. S5 to include a side view in which the “capped” BCD is more apparent (Edit > Align Viewer… > X axis).

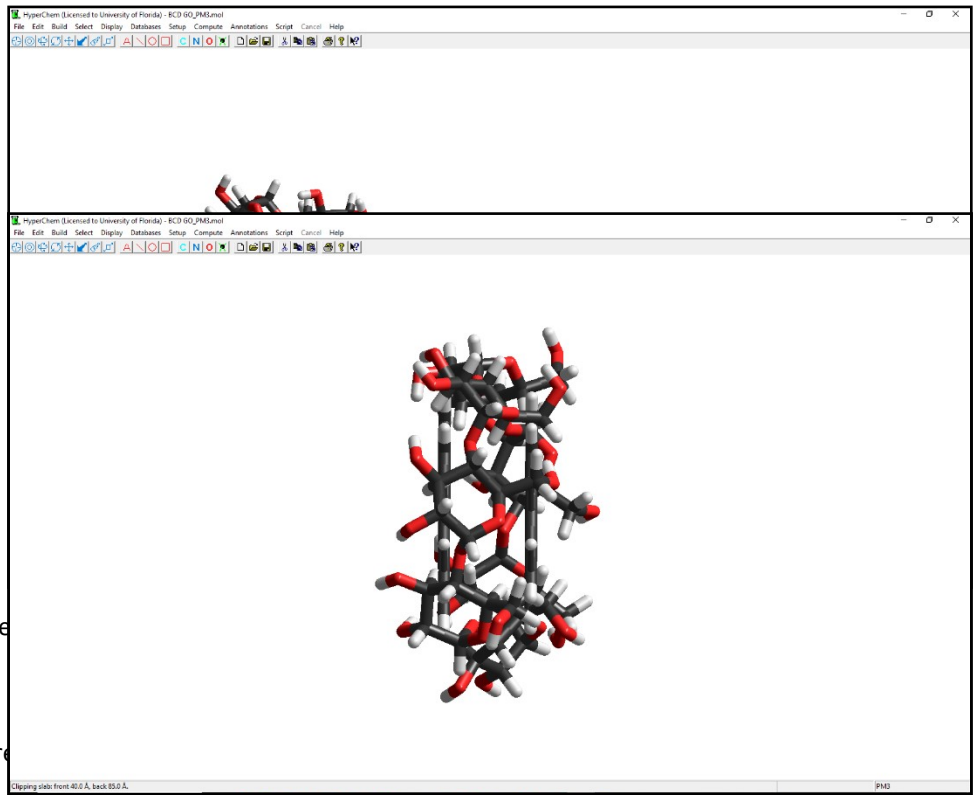


Fig. S4 The se

Fig. S5 Differe

f. The mol file is imported into SwissPDB where the van der Waal's surface of "capped" BCD is computed as **1245 Å^3** (File > Open MOL (SDF) File… then Tools > Surface > Compute).

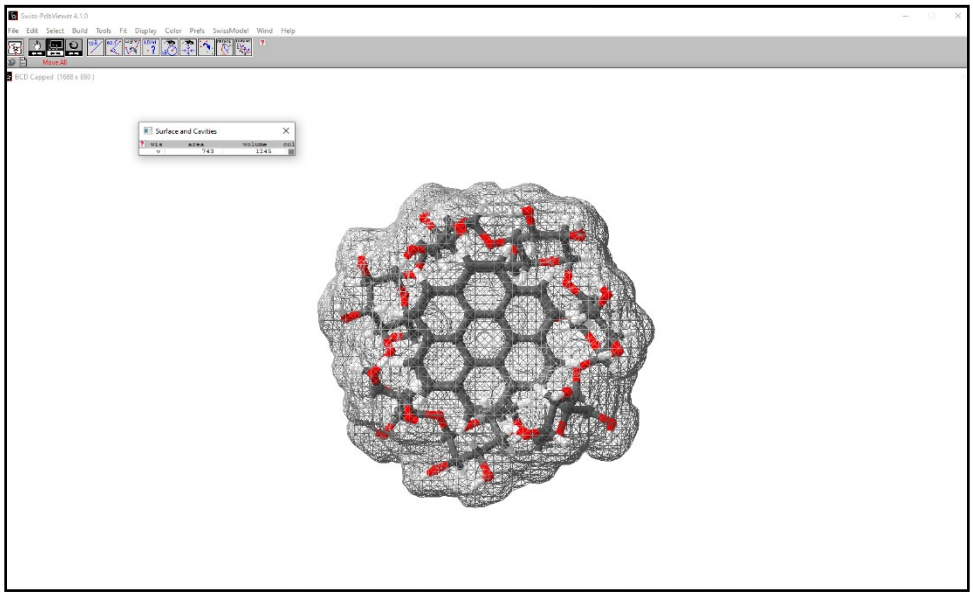


Fig. S6 Van der Waal's surface of "capped" BCD as generated in SwissPDB.

g. In a new SwissPDB window, the mol file of BCD is imported. Its van der Waal's surface is computed as **984 Å^3** (File > Open MOL (SDF) File… then Tools > Surface > Detect Cavities).

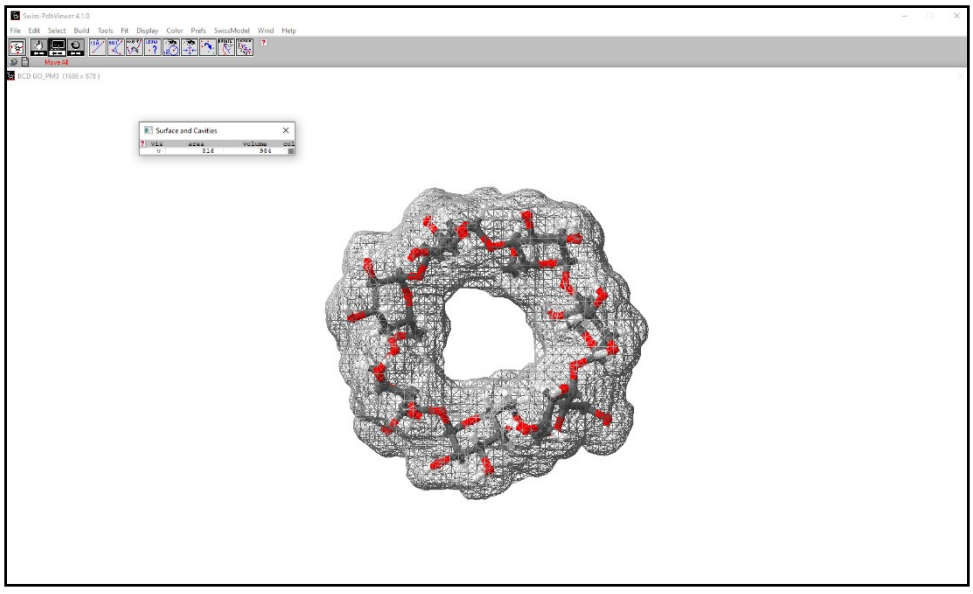


Fig. S7 Van der Waal's surface of BCD as generated in SwissPDB.

h. The cavity volume of BCD is estimated as **261 Å^3** by subtracting the volume in g (**984 Å^3**) from the volume in f (**1245 Å^3**).

Additional Notes:

1. For ACD, the coronenes are positioned at [X = 0.000, Y = 0.000, Z = -1.800] and [X = 0.000, Y = 0.000, Z = +1.500].
2. For GCD, one of the coronenes is substituted with a circumcoronene to account for its widest rim. The position of the coronene is [X = 0.000, Y = 0.000, Z = -1.800] and that of the circumcoronene is [X = 0.000, Y = 0.000, Z = +1.900].
3. In all 3 cases, the positions of the coronene and/or circumcoronene are determined arbitrarily by considering the overall structures of the "capped" CDs. Coronene and/or circumcoronene are placed close enough to the CD rims to allow for surface contacts, but without being too deep inside the CD cavities which would result in an underestimation of their cavity volumes.
4. The method described above can be extended to a variety of host molecules other than CDs.

Binding energy equation and plot

General method

Hyperchem was used to simulate the complexation between tcyDTDO and ACD, BCD, and GCD. A dielectric constant of 78.4 was applied to mimic water (Setup > Molecular Mechanics… > AMBER > Options…).[3] Following the method laid out by Liu and Guo,[4] tcyDTDO was sequentially moved along an axis that passes through the cavity of each individual CD from -10 Å to +10 Å with the origin taken as the center of the CD cavity. With each 1 Å step from the starting point to the end point, geometry optimization of the system was performed at the PM3 level and the binding energy (BE) of the minimized complex was calculated using the equation provided below.

$$\text{Binding energy (BE), } \Delta H = \text{Energy of IC} - \left(\text{Energy of host} + \text{Energy of guest}\right)$$

Equation S1. Equation used to calculate the BEs of three tcyDTDO-CD systems.

Table S1. BEs of tcyDTDO with ACD, BCD, and GCD obtained using Equation S1.

Position along *Z*-axis (Å)	**Binding energy (kcal/mol)**		
	ACD	**BCD**	**GCD**
10	-1.59	-0.689	-0.600
9	-2.20	-1.25	-1.01
8	-3.45	-2.22	-1.67
7	-5.66	-3.82	-2.71
6	-23.4	-6.09	-4.28
5	-23.2	-23.8	-6.44
4	-23.4	-27.3	-22.3
3	-23.4	-25.5	-23.4
2	-23.3	-26.5	-23.3
1	-18.1	-26.2	-22.5
0	-23.7	-29.2	-20.5
-1	6.55	-27.9	-23.1
-2	79.9	-27.4	-19.4
-3	-16.0	-25.9	-21.9
-4	-11.6	-23.7	-22.5

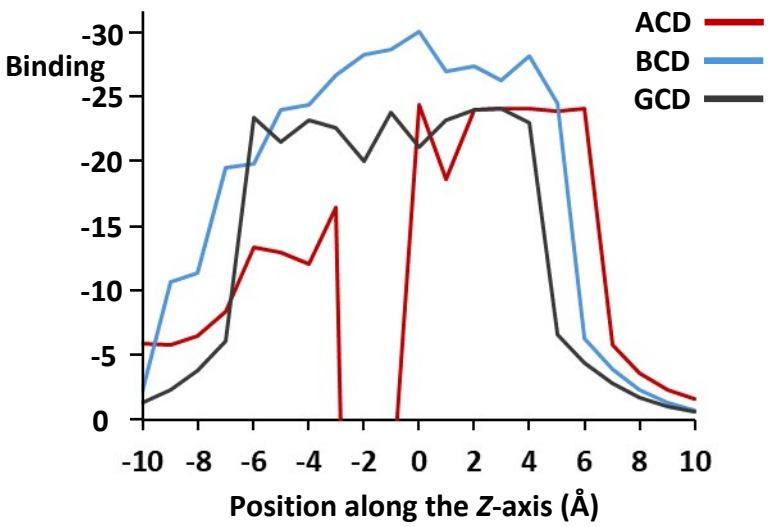


Fig. S8 BE of tcyDTDO with ACD, BCD, and GCD obtained from complexation simulations. More favorable BEs were observed with BCD and GCD.

-5	-12.6	-23.3	-20.8
-6	-13.0	-19.3	-22.7
-7	-8.15	-18.9	-5.88
-8	-6.31	-11.0	-3.71
-9	-5.61	-10.4	-2.20
-10	-5.69	-2.10	-1.29

Inclusion complex preparation by kneading

BCD hydrate and HPB were obtained from Oakwood Chemical, Inc. TcyDTDO was prepared following a literature procedure reported elsewhere by our group.[5] To prepare the tcyDTDO-BCD inclusion complex (IC), a paste was formed by mixing 1.14 g (1 mmol, MW = 1153 g/mol) of BCD with a few drops of deionized water. To the paste, 0.206 g (1 mmol, MW = 206.32 g/mol) of tcyDTDO was added and the equimolar mixture was kneaded with a mortar and pestle for about 20 minutes. Deionized water was added periodically to maintain a paste-like consistency. The resulting IC was dried in an oven at 60 °C for 12 h before characterization by ATR-FTIR.

ATR-FTIR overlays

ATR-FTIR spectra were recorded on a Perkin Elmer Spectrum 1™ instrument equipped

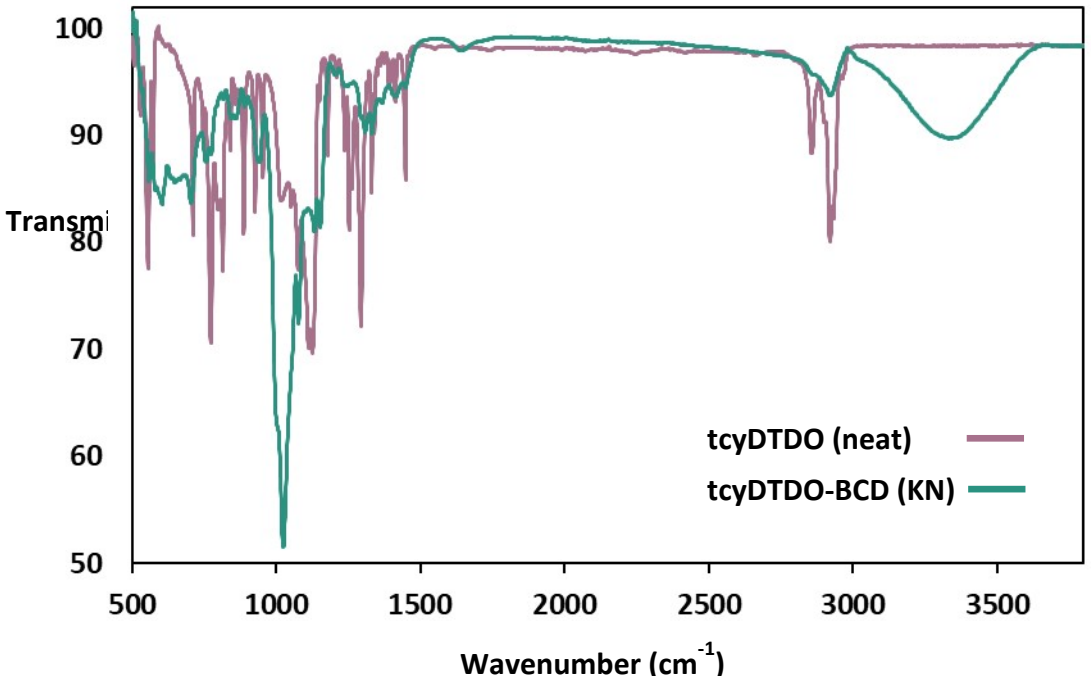
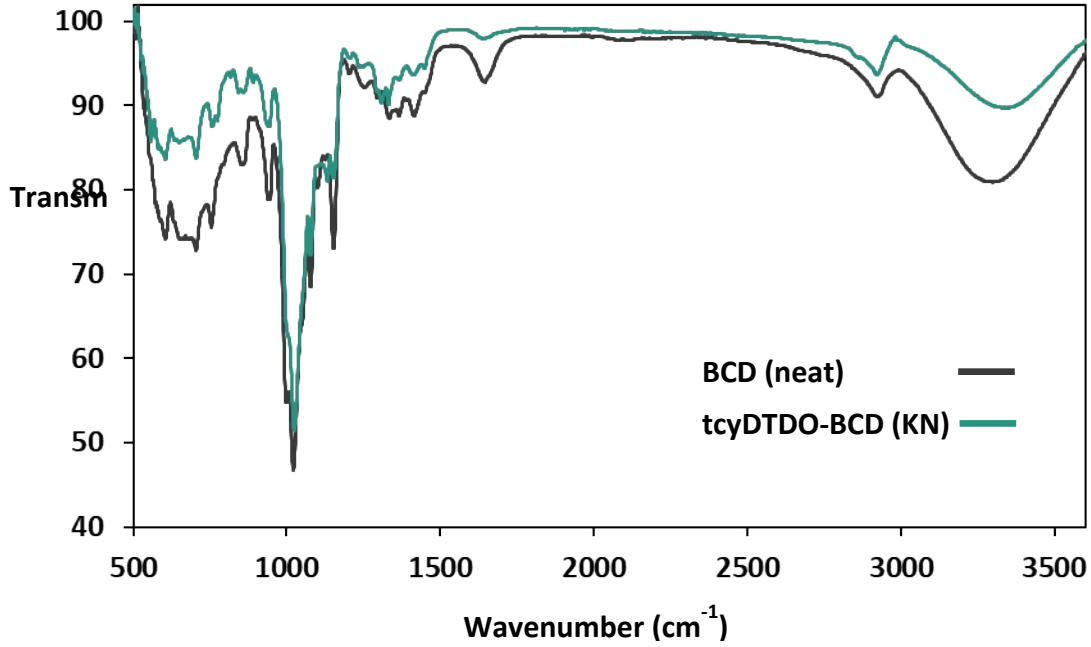


Fig. S9 ATR-FTIR overlay (in full) of neat tcyDTDO and tcyDTDO-BCD kneaded complex.



with a zinc selenide crystal tip. All samples were analyzed as solids.

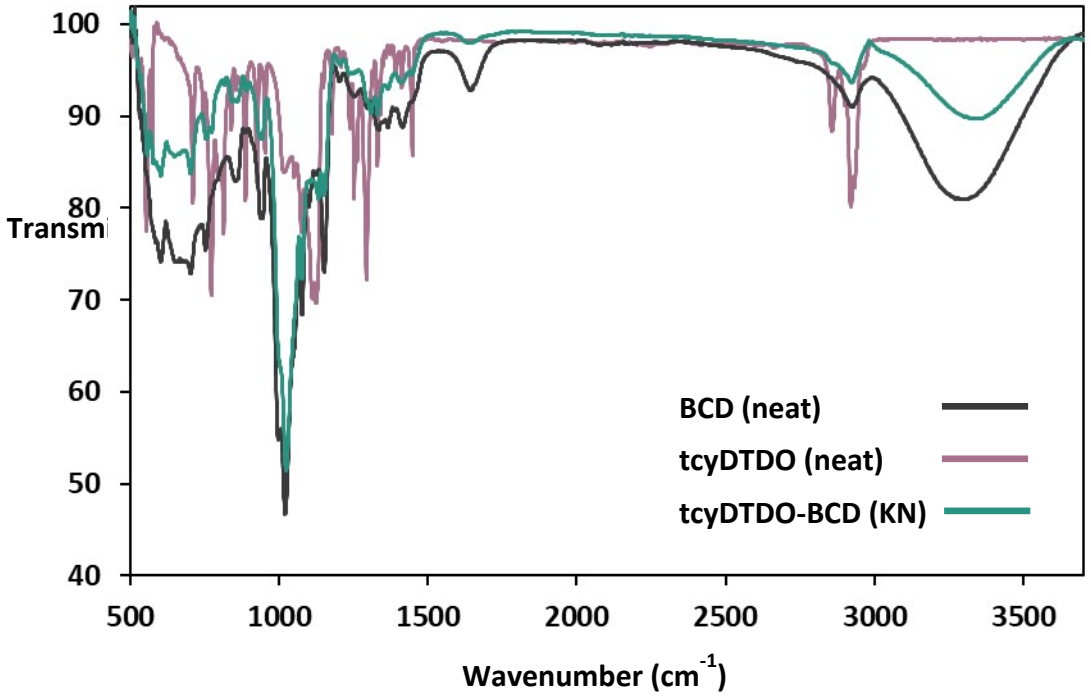


Fig. S11 ATR-FTIR overlay (in full) of neat tcyDTDO, neat BCD, and tcyDTDO-BCD kneaded complex.

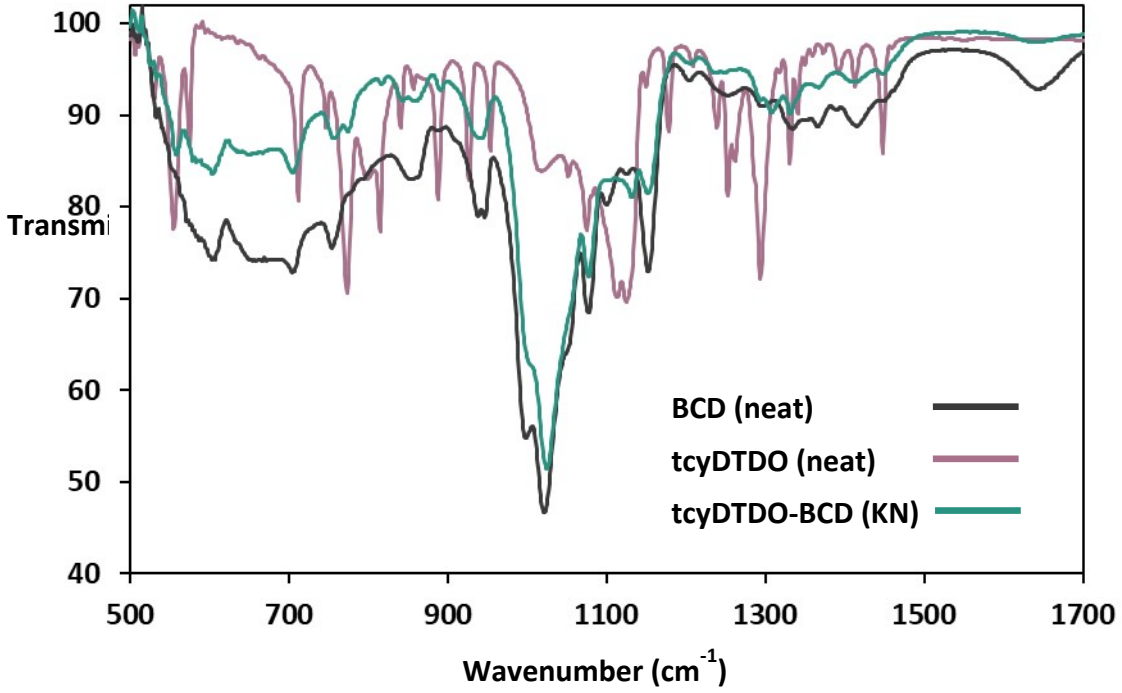


Fig. S12 ATR-FTIR overlay (focused) of neat tcyDTDO, neat BCD, and tcyDTDO-BCD kneaded complex from 500 cm^{-1} to 1700 cm^{-1}.

Phase-solubility plots

To construct the phase-solubility plots, the method laid out by Ghiviriga was followed.[6] The obtained concentrations of tcyDTDO and CDs (BCD and HPB) are tabulated below.

Table S2. Concentrations of tcyDTDO and BCD measured by NMR.

[BCD] (mM)	[tcyDTDO] (mM)
0	1.58
0.14	1.57
0.22	1.86
0.26	1.94
0.45	2.11
0.49	2.15
0.77	2.34
0.82	2.34
1.13	2.57
1.16	2.67
1.70	3.00
1.80	3.20
2.13	3.24
2.36	3.71
2.37	3.82
2.39	3.76
2.42	3.70
2.48	3.78
2.53	3.74
2.56	3.74
2.57	3.85
2.65	3.91
3.11	4.19
3.38	4.41
3.41	4.47
4.43	5.75

Table S3. Concentrations of tcyDTDO and HPB measured by NMR.

[HPB] (mM)	[tcyDTDO] (mM)
0	1.58
21.4	4.14
38.8	5.52
64.7	9.40
76.0	10.5
111	13.6
141	15.7
176	21.4
209	25.3
241	27.2
280	29.5
295	30.6
311	35.1
364	41.6
399	43.1
457	49.0
526	57.3
571	64.8
614	70.0
631	73.0
666	76.7
842	92.0
905	105
910	108
920	102
945	105
1009	117
1036	118
1148	135
1325	147

Association constants

Association constants (K_a) were calculated using the equation derived by Higuchi and Connors (Equation S2).[7]

$$\text{Association constant } (K_a)_{1:1} = \frac{\text{Slope}}{S_0\,(1 - \text{Slope})}$$

Equation S2. Equation used to calculate the association constants of two tcyDTDO-CD systems. Slope: Gradient of phase-solubility plot. S_0: Intrinsic solubility of tcyDTDO.

K_a between tcyDTDO and BCD

$$K_{a\,(\text{tcyDTDO-BCD})} = \frac{0.866}{1.58 \times 10^{-3}\,(1 - 0.866)}$$

$$= 4090\ \text{M}^{-1}$$

K_a between tcyDTDO and HPB

$$K_{a\,(\text{tcyDTDO-HPB})} = \frac{0.113}{1.58 \times 10^{-3}\,(1 - 0.113)}$$

$$= 81\ \text{M}^{-1}$$

MTT cell viability assays

General method

MDA-MB-468 cells were plated at 7,500 cells/well in 96-well plates and incubated at 37°C for 24 h. Cells were subsequently treated with BCD, HPB, tcyDTDO, tcyDTDO-BCD, and tcyDTDO-HPB for 72 h at 37 °C. Following treatment, cells were incubated with 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Biomatik, Wilmington, DE, USA) dissolved in Phosphate Buffered Saline (PBS) at 37 °C for 1 h. After removal of the MTT solution, the MTT formazan product was dissolved in 100 µl of DMSO and MTT formazan absorbance (570 and 690 nm) was measured in a plate reader. The experiment was performed in 10 replicates and the data collected is tabulated below.

SD: Standard deviation

TcyDTDO

Table S4. Percentage of live MDA-MB-468 (EGFR+) cells remaining after treatment with the indicated concentration of tcyDTDO.

Concentration (µM)	Mean	SD
0	100	8.411669
0.391	87.63282	5.939887
1.56	28.80756	4.25503
6.25	5.342385	1.036099
25	2.892562	0.678795
100	1.74144	0.25844

BCD and HPB

Table S5. Percentage of live MDA-MB-468 (EGFR+) cells remaining after treatment with the indicated concentration of BCD or HPB.

Concentration (µM)	BCD		HPB	
	Mean	SD	Mean	SD
0	100.0001	22.23628	100.0001	15.07675
0.391	100.317	9.391368	96.0174	11.8576
1.56	97.14775	5.598641	95.17258	14.15493
6.25	95.85123	7.601556	87.29768	8.837017
25	88.90765	15.50063	79.48313	8.720587
100	84.41305	13.16688	83.73739	11.35905

TcyDTDO-CD formulations

Table S6. Percentage of live MDA-MB-468 (EGFR+) cells remaining after treatment with the indicated concentration of the respective formulation.

Concentration (μM)	TcyDTDO-BCD		TcyDTDO-HPB	
	Mean	SD	Mean	SD
0	100.0001	13.19722	100.0001	13.04152
0.391	100.2384	12.64934	98.9104	16.83129
1.56	73.12214	7.715398	88.93791	10.5534
6.25	17.85684	2.469012	32.90042	8.244067
25	4.642956	0.33145	5.624662	0.618756
100	2.603939	0.394752	2.784813	0.543714

Oral pharmacokinetics in Sprague-Dawley rats

Bioanalysis and LC-MS/MS conditions

Quantitative analysis of tcyDTDO in rat plasma was performed using a Waters Acquity Class I Plus ultra-performance liquid chromatography system coupled with a Waters Xevo TQ-XS triple quadrupole mass spectrometer (UPLC-MS/MS) (Waters, Milford, MA, USA). Electrospray ionization (ESI) in positive ion mode was used for analyte detection via multiple reaction monitoring (MRM). The mass transitions monitored were *m/z* 207.16 → 109.02 for tcyDTDO and *m/z* 180.11 → 110.02 for phenacetin (internal standard). Chromatographic separation was performed on an Acquity UPLC CSH C18 column (50 × 2.1 mm, 1.7 µm) at a flow rate of 0.3 mL/min using a gradient elution with a mobile phase of (A) 0.1% formic acid in deionized water and (B) methanol. Initially, the composition was 35% B for 2.5 min, then linearly increased to 60% B over 2.5 min, rapidly returning to 35% B and maintained till 3 min. A 2 µL injection volume was used, with retention times of 2.06 min for the analyte and 1.43 min for the internal standard.

Plasma samples were analyzed over a linearity range of 2.5-400 ng/mL, with quality control standards including lower limit of quantification (LLOQ, 2.5 ng/mL), low quality control (LQC, 7.5 ng/mL), medium quality control (MQC, 180 ng/mL), and high-quality control (HQC, 340 ng/mL). TcyDTDO was extracted from plasma samples using a protein precipitation procedure. Briefly, a 20 µL aliquot of plasma was mixed with 80 µL of acetonitrile containing 0.1% formic acid and phenacetin (1 ng/mL) as the internal standard. The mixture was vortexed for 5 minutes, followed by filtration using 0.45 µm filter plates (Millipore Solvinert® plates) and centrifugation at 1500 rpm for 5 minutes. The prepared samples were then injected into the UPLC-MS/MS system for analysis.

Pharmacokinetics Study

Jugular vein catheterized male Sprague-Dawley rats (225 ± 25 g) were procured from Envigo (Indianapolis, IN, USA) and allowed a one-week acclimatization period under standard environmental conditions. All animal procedures were carried out in compliance with the Guidelines for Care and Use of Laboratory Animals of the University of Florida (UF) and approved by the UF Institutional Animal Care and Use Committee (IACUC). TcyDTDO was dosed as an aqueous suspension in sodium carboxy methyl cellulose (0.5%, w/v, NaCMC). BCD and HPB formulations were administered directly. All three systems contained an equivalent tcyDTDO dose of 9 mg/kg. Blood samples were collected at intervals of 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 18, and 24 hours after oral administration. Blood samples were subjected to centrifugation at 2000 rpm for 10 minutes to isolate the plasma, which was subsequently stored at -80°C until further analysis. Pharmacokinetic parameters of tcyDTDO were determined using Phoenix software version 8.5.2 (Certara, Princeton, NJ, USA), employing non-compartmental analysis of the plasma concentration-time data.

R: Rat

SD: Standard deviation

SEM: Standard error mean

NA: Sample not available or sample missed

TcyDTDO-BCD

Table S7. Plasma concentration of tcyDTDO measured over a 24-hour period following oral administration of the tcyDTDO-BCD formulation in Sprague-Dawley rats.

Time (h)	Plasma concentration (ng/mL)						
	R1	R2	R3	R4	Mean	SD	SEM
0.083	4514.26	7804.60	5691.26	8352.68	6590.70	1798.03	899.01
0.25	1890.48	3064.16	2344.42	4070.42	2842.37	950.67	475.34
0.5	1119.84	1292.23	782.53	1268.39	1115.75	234.87	117.44
1	270.25	393.26	320.97	487.37	367.96	94.26	47.13
2	191.42	356.72	267.35	605.34	355.21	179.92	89.96
4	37.42	116.03	45.13	8.57	51.79	45.63	22.81
6	15.70	94.55	19.10	8.37	34.43	40.33	20.16
8	12.83	24.22	24.06	7.64	17.19	8.31	4.15
12	19.00	47.62	NA	3.36	23.33	22.44	11.22
18	13.55	9.69	47.29	1.91	18.11	20.05	10.02
24	11.10	33.53	17.21	1.61	15.86	13.41	6.71

TcyDTDO-HPB

Table S8. Plasma concentration of tcyDTDO measured over a 24-hour period following oral administration of the tcyDTDO-HPB formulation in Sprague-Dawley rats.

Time (h)	Plasma concentration (ng/mL)						
	R1	R2	R3	R4	Mean	SD	SEM
0.083	1229.51	1189.14	1374.75	1192.66	1246.52	87.42	43.71
0.25	906.21	1182.86	1186.61	515.50	947.80	316.70	158.35
0.5	237.17	517.77	552.59	270.01	394.39	163.75	81.87
1	147.97	157.03	127.10	225.15	164.31	42.45	21.23
2	124.64	43.90	96.22	94.43	89.80	33.58	16.79
4	14.67	14.04	70.28	13.84	28.21	28.05	14.03
6	9.33	35.10	23.51	4.29	18.06	13.97	6.99
8	14.68	13.31	14.39	14.23	14.15	0.59	0.30
12	4.62	50.10	8.73	23.68	21.78	20.58	10.29
18	14.45	26.52	27.91	6.98	18.96	10.02	5.01
24	1.72	9.09	6.39	6.95	6.04	3.10	1.55

TcyDTDO-NaCMC

Table S9. Plasma concentration of tcyDTDO measured over a 24-hour period following oral administration of the tcyDTDO-NaCMC suspension in Sprague-Dawley rats.

Time (h)	Plasma concentration (ng/mL)						
	R1	R2	R3	R4	Mean	SD	SEM
0.083	2279.03	3247.09	3569.07	3230.30	3081.37	557.15	278.57
0.25	2002.33	2760.18	2937.27	1239.79	2234.89	777.51	388.76
0.5	805.80	1061.24	1129.40	615.37	902.95	236.98	118.49
1	388.72	278.38	281.79	314.80	315.92	51.24	25.62
2	185.39	167.55	235.14	89.75	169.46	60.35	30.17
4	32.29	47.19	30.06	20.72	32.57	10.96	5.48
6	12.16	42.89	23.88	23.55	25.62	12.74	6.37
8	11.26	30.04	21.27	24.87	21.86	7.93	3.97
12	5.80	6.21	12.61	8.91	8.38	3.14	1.57
18	23.14	19.87	19.04	15.86	19.48	2.99	1.50
24	3.82	16.15	7.14	32.93	15.01	13.03	6.51

Statistical analysis of C_{max} and P-values

Table S10. P-values computed using GraphPad Prism to assess any significant statistical difference in C_{max}.

Time (h)	HPB/BCD	HPB/NaCMC	NaCMC/BCD
0.083	0.001	0.0006	0.0098
0.25	0.0092	0.022	0.3607
0.5	0.0024	0.0124	0.2493
1	0.0076	0.0039	0.3694
2	0.0273	0.0605	0.098
4	0.4125	0.782	0.4439
6	0.4721	0.4543	0.6915
8	0.4934	0.1007	0.4468
12	0.7841	0.2454	0.4381
18	0.9417	0.9251	0.8971
24	0.2034	0.2289	0.9303

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