Molecular Modeling Based Delivery System Enhances Everolimus-Induced Apoptosis in Caco-2 Cells

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1. **Assessment of FGF7:β-CD:EV complex stability and EV release profile in Dulbecco's Modified Eagle Medium (DMEM)**

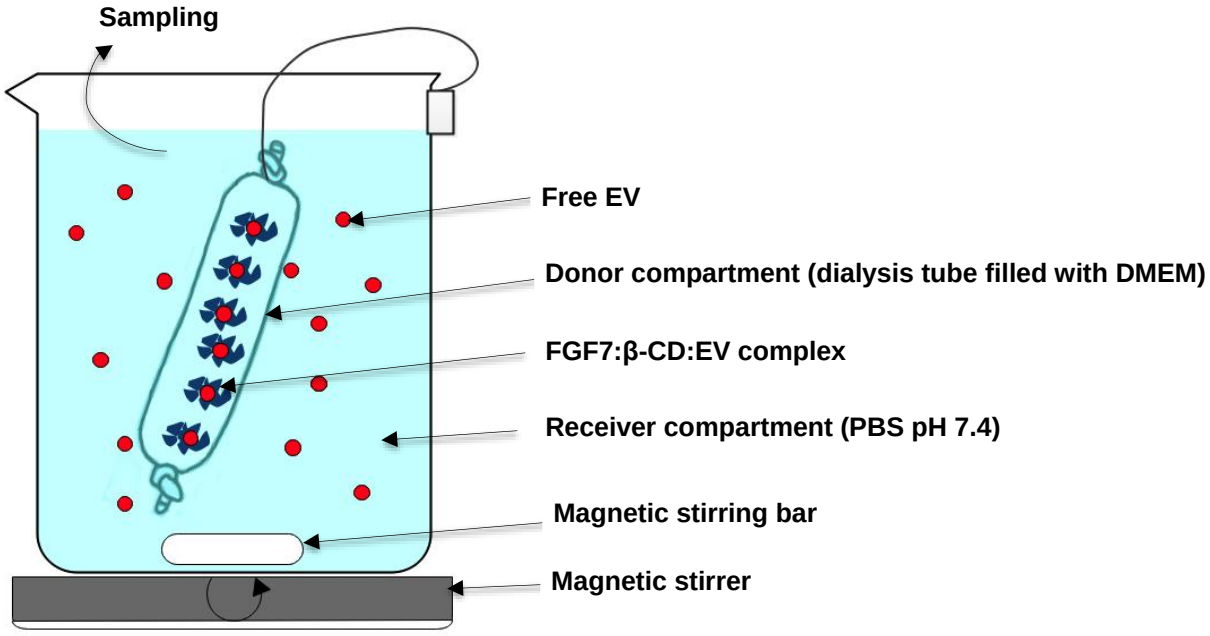

Figure S1. Dialysis tubing procedure

2. Cytotoxicity of FGF7:β-CD:EV complex on normal cells

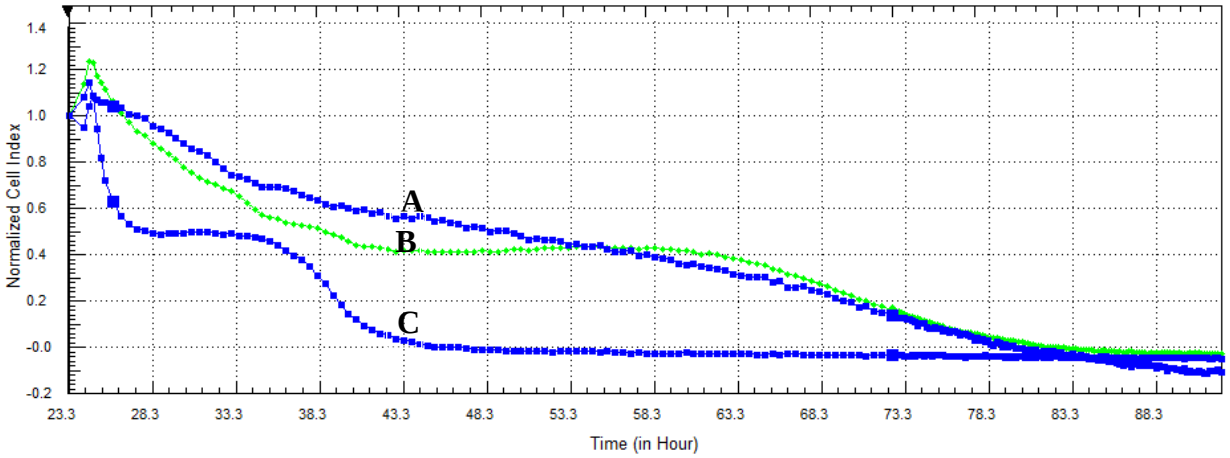

Figure S2. Cytotoxic effect of EV on FHs 74 Int cells as displayed by the RTCA DP instrument. Cells were seeded overnight to reach the log phase, then incubated with 3.26 µM EV (A), 6.52 µM EV (B), or 13.04 µM EV (C). IC_{50} value of EV on FHs 74 Int cells was found to be 19.25 ± 1.35 µM.

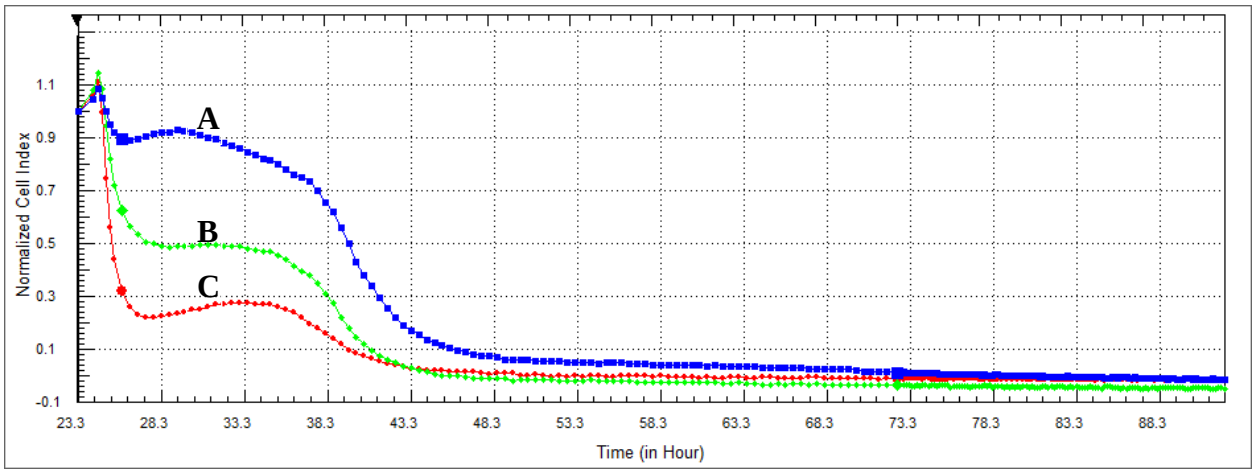

Figure S3. Cytotoxic effect of FGF7:EV on FHs 74 Int cells as displayed by the RTCA DP instrument. Cells were seeded overnight to reach the log phase, then incubated with FGF7:EV complex (contains 1.58 µM FGF7 and 3.26 µM EV) (A), FGF7:EV (contains 1.58 µM FGF7 and 6.52 µM EV) (B), or FGF7-EV (contains 1.58 µM FGF7 and 13.04 µM EV) (C). IC_{50} value of EV on FHs 74 Int cells was found to be 15.9 ± 0.95 µM.

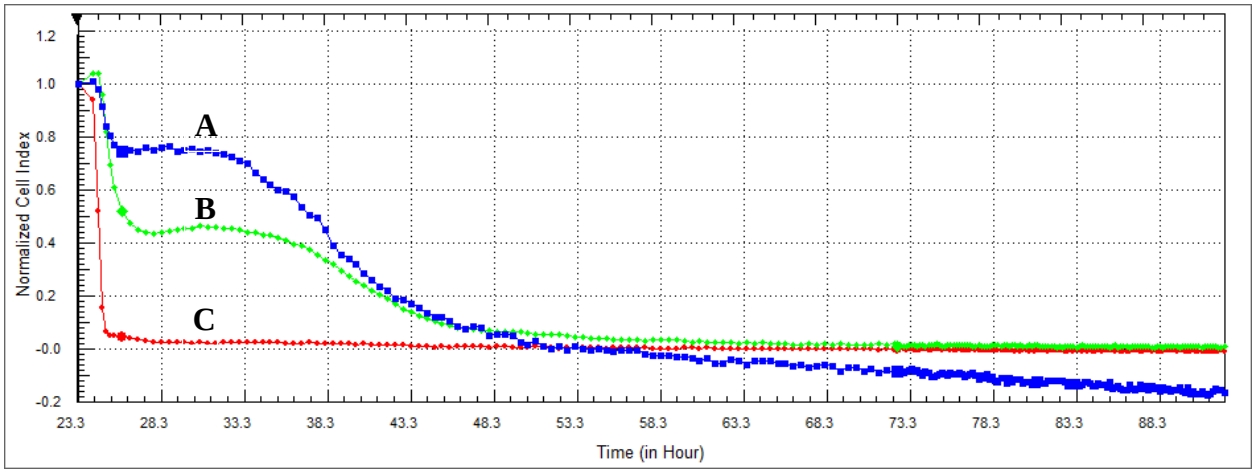

Figure S4. Cytotoxic effect of β-CD:EV on FHs 74 Int cells as displayed by the RTCA DP instrument. Cells were seeded overnight to reach the log phase, then incubated with β-CD:EV inclusion complex (contains 3.26 µM EV) (A), β-CD:EV inclusion complex (contains 6.52 µM EV) (B), or β-CD:EV inclusion complex (contains 13.04 µM EV) (C). IC_{50} value of EV on FHs 74 Int cells was found to be 16.55 ± 1.05 µM.

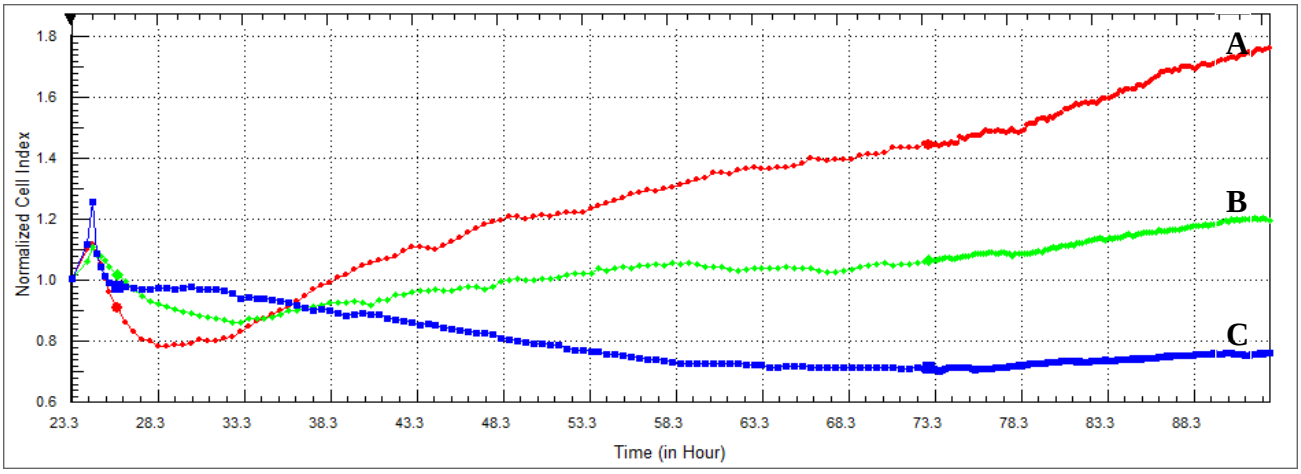

Figure S5. Cytotoxic effect of FGF7:β-CD:EV complex on FHs 74 Int cells as displayed by the RTCA DP instrument. Cells were seeded overnight to reach the log phase, then incubated with FGF7:β-CD:EV complex (contains 1.58 µM FGF7, 55.07 µM β-CD and 3.26 µM EV) (A), FGF7:β-CD:EV complex (contains 1.58 µM FGF7, 110.13 µM β-CD and 6.52 µM EV) (B) or FGF7:β-CD:EV complex (contains 1.58 µM FGF7, 220.27 µM β-CD and 13.04 µM EV) (C). IC_{50} value of EV on FHs 74 Int cells was found to be 34.11 ± 1.9 µM.

3. Assessment of FGF7:β-CD:EV complex retention in Caco-2 cells

Chromatographic conditions

The method is modified from Carpentier et al.[1] and Spandana et al. [2]. Briefly, treated cells were detached and centrifuged at 400g x 7 minutes, then 0.1 ml of tetraborate buffer (pH 9.8), 0.1 ml of EV solution (10 mg/L) as internal standard, and 1.8 ml of dichloromethane/methanol 4:l (v/v) were immediately added to the cell pellet. After a vigorous agitation, the organic phase was removed and evaporated to dryness. The dry residue was dissolved in the mobile phase: acetonitrile/double ultra-pure water 95:05 (v/v), and injected onto an Ascentis® C18 column (Supelco, Bellefonte PA, USA). The flow rate was 1 ml/min, the peaks were detected by PDA detection and wave length was set at 278 nm.

The results indicated that the penetration of EV from both samples was time-dependent, thus the accumulation was significantly increased with FGF7:β-CD:EV complex application, only 3% of free EV was detected. The retention of EV was enhanced with the complex application as well. After 24 hours of exposure, ~3.5% of free drug was detected from cells treated with complex sample, and ~7% from cells treated with EV only sample.

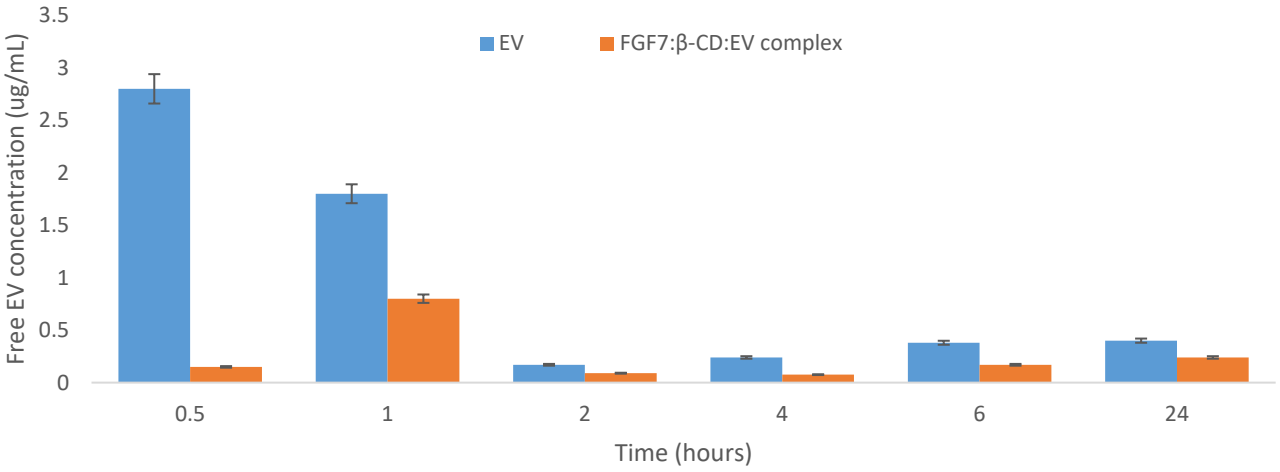

Figure S6. The influence of FGF7:β-CD complex on the time-dependent rate of Caco-2 cells uptake and retention of EV for an administered dose of 6.52 μM. The free EV concentration was measured by HPLC.

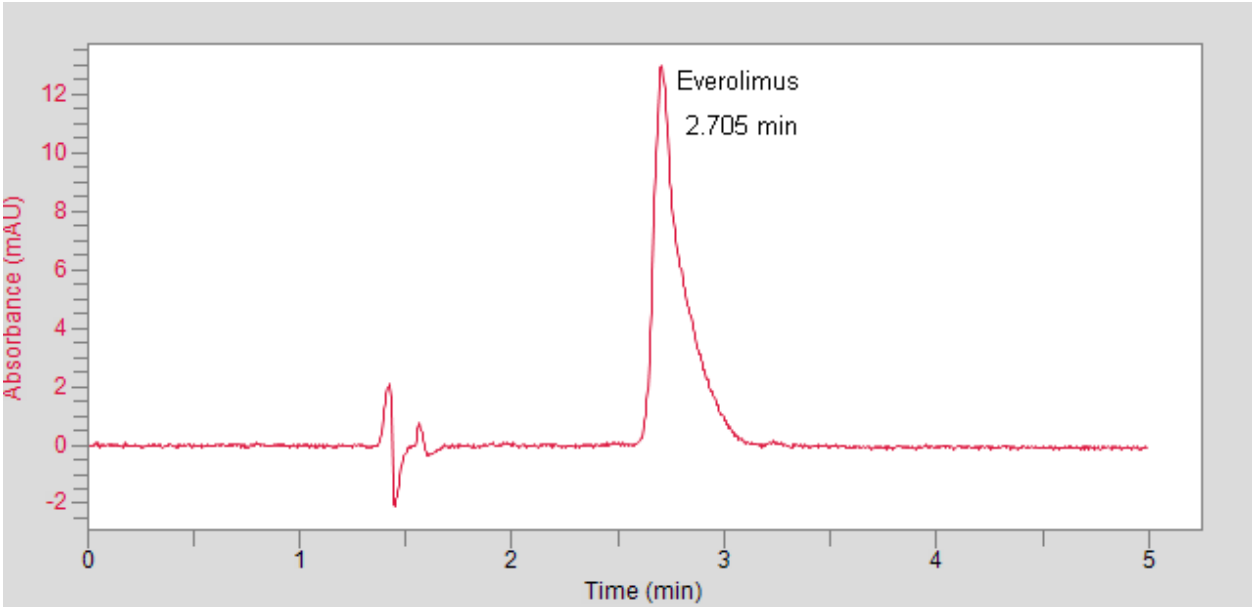

Figure S7. Standard chromatogram of EV (6.25 μg/mL).

4. Target Identification of β-CD and EV

Swiss target prediction software has been used for predicting molecular targets of β-CD and EV [3]. SMILES of β-CD and EV were obtained from PubChem (Table S1). The consensus molecular targets predicted by Swiss for β-CD and EV are listed in Table S3 and S4.

Table S1. Canonical SMILES of β-CD and EV

Name	SMILE
CD (PUBCHEM CID: 444041)	C(C1C2C(C(C(O1)OC3C(OC(C(C3O)O)OC4C(OC(C(C4O)O)OC5C(OC(C(C5O)O)OC6C(OC(C(C6O)O)OC7C(OC(C(C7O)O)OC8C(OC(O2)C(C8O)O)CO)CO)CO)CO)CO)CO)O)O)O
EV (PUBCHEM CID: 6442177)	CC1CCC2CC(C(=CC=CC=CC(CC(C(=O)C(C(C(=CC(C(=O)CC(OC(=O)C3CCCCN3C(=O)C(=O)C1(O2)O)C(C)CC4CCC(C(C4)OC)OCCO)C)C)O)OC)C)C)C)OC

Swiss predicts the molecular targets of small molecules based on their 2D and 3D similarity by comparing the query molecule to a library of 280 thousand compounds. Table S2 and S3 listed the molecular targets of β-CD and EV respectively. With high probability Swiss predicted that β-CD could target FGF-7 and FGF-10 (also known as KGF-1 and KGF-2, respectively) based on its 3D similarity to CHEMBL198643 (Figure S6) with a similarity score of 0.758 out of 1 EV targets Serine/threonine-protein kinase mTOR.

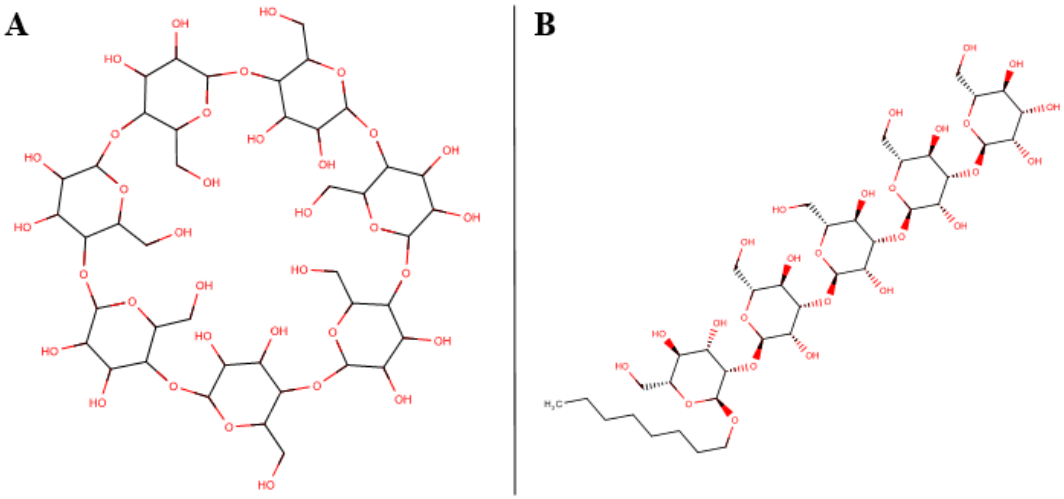

Figure S8. 2D structure of A) β-CD, B) CHEMBL198643

It has been reported that CHEMBL198643 strongly binds with FGF-1 and 2 [4]. Based on the shape-similarity theory which states that molecules possessing similar 3D structure might exhibit analogous biological activity, we thought that β-CD would be able to binds to fibroblast growth factors. This is could be supported by the strong affinity of a similar structure (*β-CD* Tetradecasulfate) toward FGF [5]. Heparin is essential for FGF to generate a biological response through binding to FGF receptor. Heparin functions by binding to several FGF molecules forming FGF oligomerization. Then the FGF-heparin complex bind to couple of FGFRs, this leads to FGFR dimerization which

activates tyrosine kinase pathway. A study showed that synthetic heparin analog which can only bind to one FGF blocks the dimerization of FGFR, thus stopping its activation. We propose that β-CD antagonizes the action of heparin by binding to only one FGF, therefore it cannot induce FGF oligomerization thus preventing FGFR dimerization and activation [6, 7].

5. Binding ability of β-CD and EV to FGF7

Molecular docking was performed via the molecular operating environment (MOE.2014) software for β-CD and EV in the heparin binding site of basic fibroblast growth factor (1BFB.pdb) [8] with scoring affinity London dG and GBVI/WSA dG. The obtained docking affinity scores are shown in Figure S9.

	mol	rseq	mseq	S	rmsd_refine	E_conf	E_place	E_score1	E_refine	E_score2
1	6442177	1	1	-5.0092	5.3896	249.3156	-27.0249	-8.0561	-17.0150	-5.0092
2	6442177	1	1	-4.8737	3.2159	238.9634	-41.8554	-8.3144	-14.0658	-4.8737
3	6442177	1	1	-4.8022	1.6303	256.8998	-21.7023	-8.5919	-15.1917	-4.8022
4	6442177	1	1	-4.7126	2.8163	245.0996	-23.7716	-8.0905	-13.0527	-4.7126
5	6442177	1	1	-4.7104	2.2270	238.7883	-38.0680	-9.0713	-12.1594	-4.7104
6	6442177	1	1	-4.6170	2.7003	254.6145	-3.1844	-7.9129	-17.1669	-4.6170
7	6442177	1	1	-4.4615	1.7729	238.0406	-30.6175	-9.5801	-14.7607	-4.4615
8	6442177	1	1	-4.2688	2.2017	237.3359	-54.2724	-8.1642	-11.7695	-4.2688
9	6442177	1	1	-4.0870	4.2871	247.2750	-40.7168	-8.3377	-8.9479	-4.0870
10	6442177	1	1	-4.0772	2.0780	256.3575	7.1687	-8.6806	-7.7938	-4.0772
11	444041	1	2	-5.2439	1.9786	624.7195	-64.3874	-9.1450	-18.8291	-5.2439
12	444041	1	2	-4.8375	2.8882	621.0886	-7.2920	-9.5388	-15.1178	-4.8375
13	444041	1	2	-4.5602	2.9452	615.8110	-78.4285	-9.1247	-11.2252	-4.5602
14	444041	1	2	-4.1812	2.3501	631.4261	-66.3765	-9.6591	-9.7925	-4.1812
15	444041	1	2	-4.0467	4.3664	631.7632	-51.2175	-9.3040	-10.8069	-4.0467
16	444041	1	2	-4.0261	3.1508	625.8408	-50.4002	-10.1711	-11.2005	-4.0261
17	444041	1	2	-3.6537	1.6517	623.4816	-80.9887	-9.6476	-9.3564	-3.6537
18	444041	1	2	-3.6457	2.4040	617.8505	-61.2162	-9.5400	-8.0208	-3.6457
19	444041	1	2	-3.6078	3.0788	624.0917	-39.1023	-9.8357	-9.6159	-3.6078
20	444041	1	2	-3.5234	2.3457	624.4732	-56.9427	-9.2841	-10.6297	-3.5234

Figure S9. Docking scores for binding ability of β-CD (PubChem CID: 444041) and EV (PubChem CID: 6442177) to FGF7, obtained by MOE.2014 software.

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