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Synthesis and biological evaluation of anti-cancer agents that selectively inhibit Her2 over-expressed breast cancer cell growth via down-regulation of Her2 protein

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ABSTRACT

Compound JCC76 selectively inhibited the proliferation of human epidermal growth factor 2 (Her2) over-expressed breast cancer cells. In the current study, a ligand based structural optimization was performed to generate new analogs, and we identified derivatives **16** and **17** that showed improved activity and selectivity against Her2 positive breast cancer cells. A structure activity relationship (SAR) was summarized. Compounds **16** and **17** were also examined by western blot assay to check their effect on Her2 protein. The results reveal that the compounds could decrease the Her2 protein, which explains their selectivity to Her2 over-expressed breast cancer cells. Furthermore, the compounds inhibited the chaperone activity of small chaperone protein that could stabilize Her2 protein.

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About 25–30% of breast cancer patients have human epidermal growth factor 2 (Her2) over-expressed tumors, and the tumor cells depend on the Her2 pathway to proliferate.[1] High expression of Her2 protein in tumors results in constitutive activation of the receptor and cell growth.[2,3] It has been well-documented that patients with over-expressed Her2 are associated with increased disease recurrence, worse prognosis and lower survival.[4] Targeting the extracellular domain of Her2 receptor could result in an efficient inhibition of breast cancer cell proliferation.[1,2,5,6] In addition, inhibition of intracellular signaling pathways of Her2 downstream could lead to suppression of cancer cell growth as well.[7] Currently, there are two types of drugs that target Her2 over-expressed cancer. The first group is Her2 monoclonal antibody drugs such as trastuzumab approved by FDA in 1998; the second type is intracellular tyrosine kinase inhibitors such as lapatinib approved in 2007.[8,9]

Although these drugs showed great efficiency in clinic for Her2 over-expressed breast cancer patients, resistance has been reported in patients with long term trastuzumab treatment.[9] There are multiple reasons for the resistance, and further increased Her2 expression in the cancer cells after the treatment is one of the resistant mechanisms.[10] Researchers used different strategies to reduce the Her2 level, and found that the cancer cells regained the sensitivity to trastuzumab.[10] Therefore, new agents that can decrease the amount of Her2 in breast cancer cells could be used for the Her2 over-expressed breast cancer treatment, and may also have the potential to overcome trastuzumab resistance.[11–13]

Our goal is to develop new anti-cancer compounds that could selectively inhibit the growth of Her2 over-expressed breast cancer cells, and then investigate the molecular mechanism of the pharmacological activity. Previously we identified a small molecule JCC76 that showed selectivity to inhibit the growth of Her2 over-expressed breast cancer cells.[14] In this study, structural optimization was performed to improve the biological activity and the selectivity of the lead compound (Fig. 1).

Compound JCC76 has been found to selectively inhibit the growth of SKBR-3 cells with an IC_{50} of 1–3 μM, and IC_{50}s of 20–25 μM to MCF-7 cells and MDA-MB-231 cells.[14] JCC76 was also found to inhibit a small chaperone protein heat shock protein 27 K_D (HSP27).[15] It has been reported that Her2 is a client protein of HSP27. The inhibition of HSP27 by JCC76 may affect the function of Her2, which explains the selectivity of JCC76 to Her2 over-expressed SKBR-3 cells.[10,16] To improve the potency, selectivity and ligand efficacy of JCC76, we further optimized the structure of this lead. Based on the structure activity relationship (SAR) summarized before, we either maintained the 2,5-dimethylbenzyl moiety or changed it to 2,5-dimethoxybenzyl group (Fig. 1),[15,17,18]

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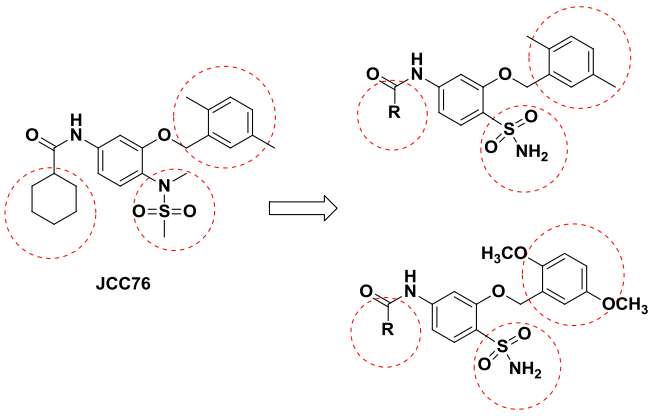


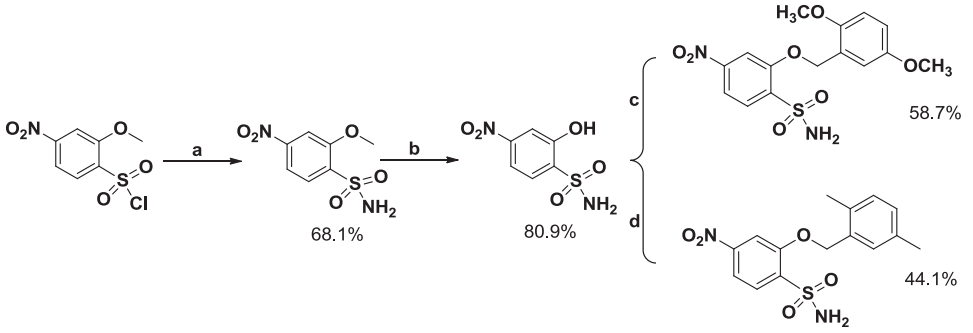
Fig. 1. Lead optimization of JCC76 to improve the ligand efficiency and biological activity.

followed by modification of the sulfonamide moiety via an orientation shift and removal of two methyl groups. These changes could increase the ligand efficacy and solubility. In addition, the amide moiety was constructed with various substituted benzamide to explore what the best functional group could be for this moiety. The synthesis of the new analogs is described in Schemes 1 and 2.

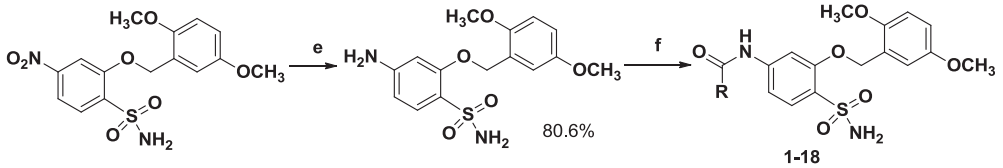
These new compounds were synthesized using methods adapted from previous studies.[15,17–20] Since the sulfonamide moiety was flipped in the new analogs compared to JCC76, the construction of the sulfonamide is different to previous synthetic methods. Twenty-three final compounds were synthesized.

The new derivatives were then examined for the potency and selectivity on the growth inhibition of three breast cancer cell lines including SKBR-3, MCF-7, and MDA-MB-231. SKBR-3 cells are Her2 positive and estrogen receptor (ER) negative, while MCF-7 cells are Her2 negative and ER positive, MDA-MB-231 cells are Her2 and ER negative. The activity of the compounds is summarized in Table 1.

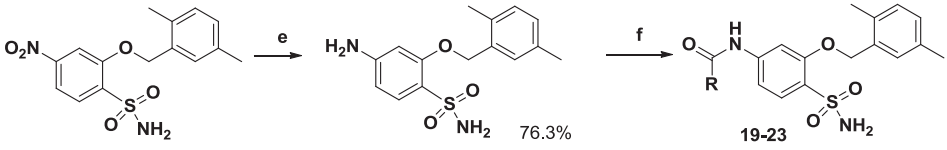
The IC_{50}s of the cell growth inhibition of the compounds range from 0.13 μM to 25.69 μM for SKBR-3 cells, 1.18 μM to 60.49 μM for MCF-7 cells, and 0.27 μM to 38.99 μM for MDA-MB-231cells. The selectivity is calculated by dividing the IC_{50}s of the compounds from different cell lines (Table 1). Most compounds exhibited higher growth inhibition in SKBR-3 cells compared to MCF-7 cells and MDA-MB-231cells, as indicated by the selective index (>1). For SKBR-3 cells, SAR analysis suggests that the benzamide group of



Scheme 1. (a) NH_3, H_2O, THF; (b) CH_2Cl_2, BBr_3; (c) 2,5-dimethoxybenzyl chloride, K_2CO_3, DMF; (d) 2,5-dimethylbenzyl chloride, K_2CO_3, DMF.



1. R=3-methoxyphenyl (71.7%)
2. R=3-methylphenyl(36.7%)
3. R=3-iodophenyl(46.3%)
4. R=3-bromophenyl(33.2%)
5. R=3-cyanophenyl(52.4%)
6. R=4-iodophenyl(37.5%)
7. R=2,4-trifluoromethylphenyl (24.3%)
8. R=4-trifluoromethoxyphenyl(61.3%)
9. R=3-cholophenyl(52.0%)
10. R=4-ethylphenyl (56.0%)
11. R=4-cyanophenyl (31.3%)
12. R=3-nitro 4-cholophenyl(52.1%)
13. R=3-ethoxyphenyl (52.5%)
14. R=4-bromophenyl (53.3%)
15. R=3,5-trifluoromethylphenyl(30.0%)
16. R=2-napthyl(52.5%)
17. R=4-methoxyphenyl (38.6%)
18. R=cyclohexanyl(77.0%)



19. R=4-iodophenyl(58.3%)
20 R=3-iodophenyl(55.8%)
21. R=cyclohexanyl(53.7%)
22. R=4-methoxyphenyl(64.2%)
23 R=4-bromophenyl(58.1%)

Scheme 2. (e) $FeCl_3$, Zn, DMF/H_2O; (f) RCOCl, K_2CO_3, 1,4-dioxane.

these compounds is critical for the biological activity. The bulky electron-donating substitutes such as iodo group on the benzamide moiety overall enhance the activity, as indicated by compounds **3** and **6**. The electron-withdrawing groups such as trifluoromethyl harm the activity, as indicated by compounds **7** and **15**. In terms of the 2,5-dimethoxybenzyl and 2, 5-dimethylbenzyl moiety, evidence shows that the methoxy contributes more to the cell growth inhibition than the 2,5-dimethybenzyl analogs, because compounds **19**–**23** are relatively less potent compared to the corresponding di-methoxy analogs. Overall, compounds **16** with a napthyl moiety and **17** with a 4-methoxyphenyl moiety showed the best potency and selectivity to SKBR-3 cells.

Table 1
Comparison of the growth inhibitory effects of the new analogs on different breast cancer cell lines.

Entry	IC_{50} (μM) (SKBR-3)	IC_{50} (μM) (MDA-231)	IC_{50} (μM) (MCF-7)	Selectivity MDA231/SKBR-3	Selectivity MCF-7/SKBR-3
1	4.03 ± 2.47	7.95 ± 4.34	4.1 ± 0.22	2.0	1.0
2	3.44 ± 1.35	15.92 ± 2.56	8.02 ± 1.65	4.6	2.3
3	1.09 ± 0.97	4.58 ± 1.7	5.25 ± 1.53	4.2	4.8
4	4.38 ± 1.25	23.01 ± 7.67	10.52 ± 0.69	5.3	2.4
5	25.07 ± 6.64	36.74 ± 25.02	14.7 ± 3.25	1.5	0.6
6	0.52 ± 0.15	1.63 ± 0.61	2.26 ± 0.36	3.1	4.4
7	25.69 ± 11.03	60.49 ± 23.83	38.99 ± 14.65	2.4	1.5
8	3.14 ± 1.03	4.08 ± 1.1	5 ± 1.76	1.3	1.6
9	3.14 ± 0.46	6.91 ± 3.42	9.01 ± 4.48	2.2	2.9
10	6.84 ± 3.18	7.44 ± 2.25	6.44 ± 0.4	1.1	0.9
11	6.25 ± 1.28	13.5 ± 7.83	21.71 ± 0.69	2.2	3.5
12	1.97 ± 0.4	3.7 ± 2.02	2.61 ± 0.14	1.9	1.3
13	0.71 ± 0.34	1.54 ± 0.94	2.87 ± 0.31	2.2	4.0
14	0.67 ± 0.15	2.22 ± 0.85	0.63 ± 0.17	3.3	0.9
15	15.81 ± 6.98	15.77 ± 7.39	24.58 ± 3.14	1.0	1.6
16	0.13 ± 0.06	1.18 ± 0.86	0.27 ± 0.09	9.1	2.1
17	2.09 ± 0.11	12.8 ± 8.98	3.38 ± 0.62	6.1	1.6
18	4.17 ± 0.78	10.79 ± 1.36	4.91 ± 0.4	2.6	1.2
19	3.01 ± 0.49	6.63 ± 0.62	4.44 ± 0.71	2.2	1.5
20	15.74 ± 5.35	48.88 ± 15.8	11.9 ± 1.17	3.1	0.8
21	15.05 ± 3.89	10.29 ± 0.72	9.7 ± 2.16	0.7	0.6
22	2.94 ± 0.75	6.5 ± 0.77	3.72 ± 0.6	2.2	1.3
23	11.22 ± 1.16	20.99 ± 0.81	10.18 ± 0.41	1.9	0.9

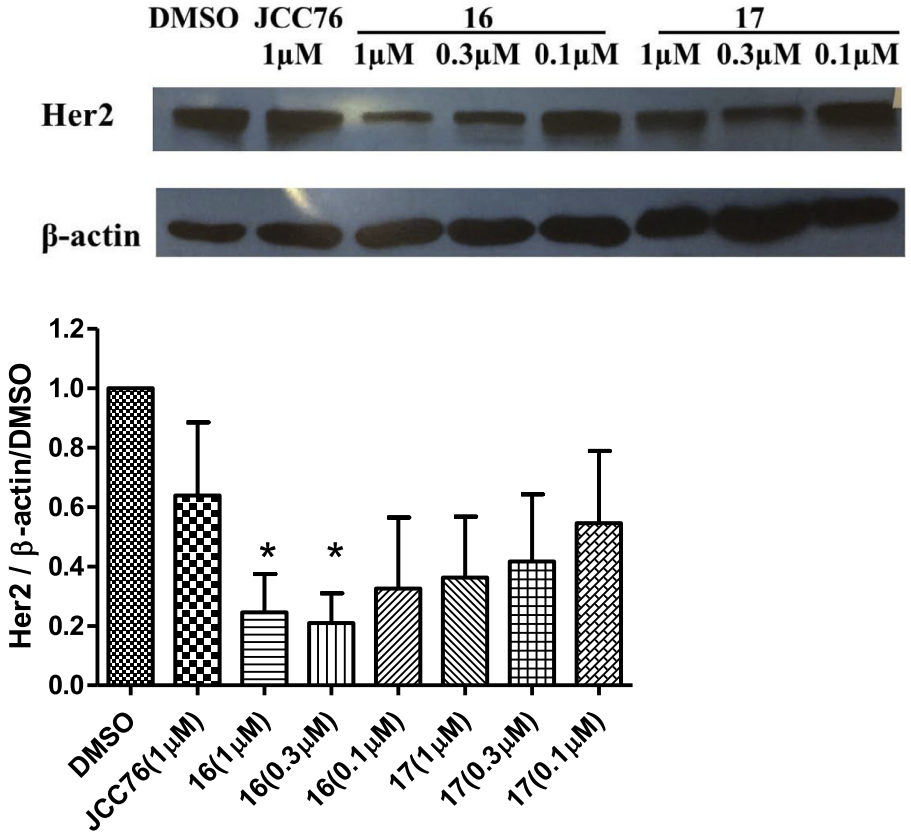


Fig. 2. Effect of compounds **16** and **17** on Her2 protein. SKBR-3 cells were treated with DMSO, JCC76 (1 μM), compounds **16** (0.1, 0.3, 1 μM) and **17** (0.1, 0.3, 1 μM) for 48 h. Level of Her2 was analyzed by Western blot of cell extracts with specific antibodies. The bands of Her2 were quantified using ImageJ (NIH) and normalized to β-actin. The results are representative of three independent experiments. $^{*}p < .05$ with unpaired t test, compound **16** vs JCC76.

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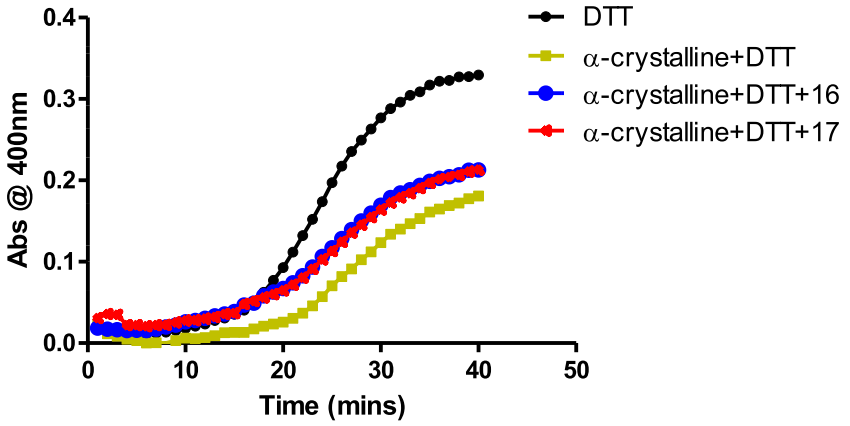

Fig. 3. α-Crystalline lost partial activity to prevent DTT induced insulin aggregation in the presence of compounds **16** (10 μM) and compound **17** (10 μM). The kinetics of the DTT-induced insulin aggregation was monitored in the absence of a chaperone protein, or in the presence of a chaperone protein without or with compounds. The mixture of insulin and DTT with or without other components in the assay buffer was incubated for 45 min at 37 °C and the absorbance at 400 nm was measured. The compounds at this concentration did not interfere with DTT and insulin interaction. The results are the representative of three independent experiments.

Based on the selectivity and potency to Her2 over-expressed SKBR-3 cells, compounds **16** and **17** were chosen for further investigation. JCC76 was demonstrated to be a small chaperone inhibitor, and also selectively inhibited SKBR-3 cell growth.[14,15] Since Her2 is a client protein of the small chaperones and is stabilized by the chaperone function, we hypothesize that JCC76 may be able to increase the degradation of Her2 via inhibition of small chaperone protein. The hypothesis is based on the fact that chaperone inhibitors could induce the degradation of the client proteins of the chaperones.[21–23] SKBR-3 cells were treated with JCC76, compounds **16** and **17** for 48 h, and the Her2 level was determined by western blot assay. As exhibited in Fig. 2, Her2 level was decreased by these new derivatives, and compounds **16** and **17** showed improved activity compared to JCC76, particularly when compound **16** was tested at 1.0 and 0.3 μM concentrations (Fig. 2). The results demonstrate that the structural optimization increased the potency and selectivity of JCC76, suggesting that the targeting effect of the compound was significantly increased.

To examine if the new compounds could interfere with the chaperone function of small chaperone proteins, an *in vitro* chaperone assay was performed. As indicated in Fig. 3, compounds **16** and **17** inhibited the protective function of small chaperone α-crystalline against Dithiothreitol (DTT) induced insulin denaturing and aggregation.[24] The results demonstrate that the new analogs retained the chaperone inhibition of the lead compound. It has been reported that Her2 protein is a client protein of small chaperone protein HSP27, and inhibition of the small chaperone could induce Her2 degradation.[10] However, it is still unclear if the chaperone inhibition by compounds **16** and **17** is the only mechanism for the decreased Her2 in SKBR-3 breast cancer cells. Further investigation is needed to determine if other possible mechanisms are involved as well.

In brief, to develop new drug candidates that could selectively target Her2 positive breast cancer cell growth, we generated 23 new derivatives based on JCC76 as a lead. Using three breast cancer cell lines as the evaluation model, the compounds were examined with cell growth assay. Two compounds **16** and **17**[25] showed good potency and selectivity against the growth of Her2 over-expressed SKBR-3 cells compared to two other cell lines. Compounds **16** and **17** also decreased the level of Her2 protein in SKBR-3 cells, which is speculated to be one of the main mechanisms of the selectivity of the compounds. In addition, the compounds inhibited the chaperone activity of α-crystalline, suggesting that they are potential small chaperone inhibitors. The biological assays in the current study are included in the Refs. 26–28.

Acknowledgements

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25. *N*-(3-((2,5-dimethoxybenzyl)oxy)-4-sulfamoylphenyl)-1-naphthamide(16). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.606 (1H, s), 8.110 (1H, d, J = 9.0 Hz), 8.073 (1H, s), 8.040 (2H, s), 7.845 (1H, s), 7.768 (1H, d, J = 8.6 Hz), 7.663 (2H, t, J = 6.1 Hz), 7.606 (1H, d, J = 8.6 Hz), 7.280 (1H, s), 7.007 (1H, d, J = 8.8 Hz), 6.877 (1H, d, J = 8.3 Hz), 5.2720 (2H, s), 3.825 (3H, s), 3.714 (3H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.466, 155.557, 153.721, 150.835 144.635, 134.893, 132.490, 132.208, 129.499, 128.802, 128.734, 128.596, 128.520, 128.180, 127.428, 126.778, 125.479, 124.869, 114.713, 113.988, 112.169, 111.562, 105.108, 65.578, 56.282, 55.806; DUIS-MS calculated for $C_{26}H_{24}N_2O_6S$, [M−H]−: 491.15, found 491.1; Purity: 98.7% *N*-(3-((2,5-dimethoxybenzyl)oxy)-4-sulfamoylphenyl)-4-methoxybenzamide(17). ^{1}H NMR (400 MHz, DMSO-d_6) 10.468 (1H, s), 7.807 (1H, s), 7.742 (1H, d, J = 8.4 Hz), 7.520 (4H, m), 7.264(1H, s), 7.200 (1H, d, J = 7.6 Hz), 7.003 (1H, d, J = 9.0 Hz), 6.932 (2H, s), 6.870 (1H, d, J = 6.8 Hz), 5.248 (2H, s), 3.856 (3H, s), 3.817 (3H, s), 3.711 (3H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.116, 159.700, 155.520, 153.721, 150.849, 144.431, 136.287, 130.136, 128.739, 126.831, 125.453, 120.401, 118.111, 114.726, 114.041, 113.555, 112.199, 111.603, 105.145, 65.553, 56.290, 55.867, 55.807; DUIS-MS calculated for $C_{23}H_{24}N_2O_7S$, [M−H]−: 471.14, found 471.0; Purity: 98.1%.

26. *Cell growth assay:* The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2*H*-tetrazolium bromide (MTT) cell viability assay was used to examine the growth inhibitory effect of the analogs on the three breast cancer cell lines in four replications. 5000 cells per well were seeded with RPMI1640 medium in 96-well flat-bottomed plates for 24 h and were then exposed to various concentrations of test compounds dissolved in DMSO (final concentration 0.1%) in medium for 48 h. Controls received DMSO at a same concentration as that in drug-treated cells. Cells were incubated in 200 μL of 0.5 mg/ml of MTT reagent diluted in fresh media at 37 °C for 2 h. Supernatants were removed from the wells, and the reduced MTT dye was solubilized with 200 μL/well DMSO. Absorbance at 570 nm was determined on a SpectraMax Plus384 spectrophotometer (Molecular Devices). Data obtained with quadreplication were normalized and fitted to a dose–response curve using GraphPad Prism v.5 (GraphPad).
27. Western blot assay: SKBR-3 cells were treated with different compounds at indicated concentrations respectively for 48 h. Total cell lysates were extracted using M-PER reagent (Pierce). Equal amounts of proteins (50 μg) samples were separated on 12% SDS-polyacrylmide gel and transferred onto a polyvinylidene diflouride (PVDF) membrane (Pall Cooperation, FL.). After blocking for 1 h, the membrane was incubated in PBST containing 5% BSA and primary antibody specific to Her2 or β-actin (Cell signaling, MA) overnight at 4 °C. HRP-conjugated anti-rabbit IgG or anti-mouse IgG (Cell signaling, MA) were used as secondary antibody and incubated at room temperature for 1 h. The membrane was incubated in ECL plus reagent (GE health) and then exposed to hyper film. The results are based on three independent experiments, and only one of the representative bands is presented.
28. Alpha-crystalline chaperone activity assay: 24 μL 1 mg/ml insulin stock solution was added to the single well of 384 well plate, 3 μL 5 mg/ml alpha-crystalline, 71 μL PBS buffer with appropriate concentration of compound dissolved inside were added as well. The mixture was thoroughly mixed and incubated at 37 °C for 5 min, whereupon 2 μL of 1 M DTT in water was added to initiate the insulin aggregation. The absorbance (A) at 400 nm was monitored over 45 min using a plate reader. A mixture of insulin in the absence or presence of alpha-crystalline with 0.1% DMSO was used as control.