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Graphical Abstract

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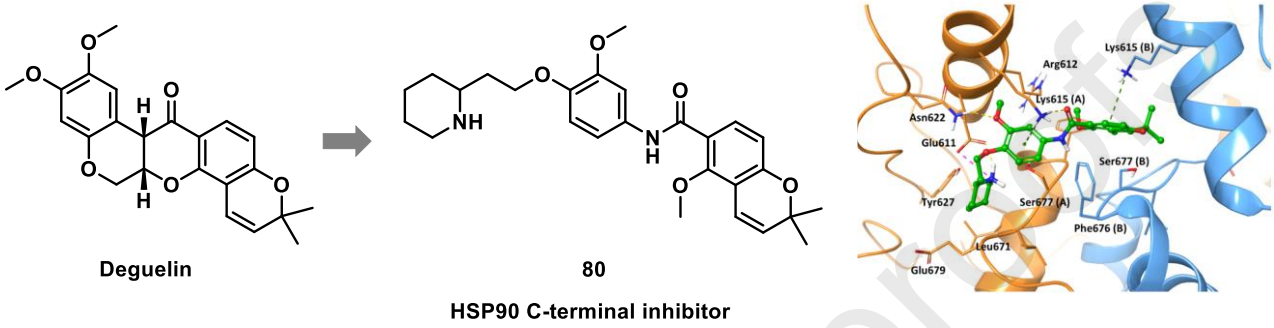

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ABSTRACT

A series of *O*-substituted analogues of the B,C-ring truncated scaffold of deguelin were designed as C-terminal inhibitors of heat shock protein 90 (HSP90) and investigated as novel antiproliferative agents against HER2-positive breast cancer. Among the synthesized compounds, compound **80** exhibited significant inhibition in both trastuzumab-sensitive and trastuzumab-resistant breast cancer cells, whereas compound **80** did not show any cytotoxicity in normal cells. Compound **80** markedly downregulated the expression of the major client proteins of HSP90 in both cell types, indicating that the cytotoxicity of **80** in breast cancer cells is attributed to the destabilization and inactivation of HSP90 client proteins and that HSP90 inhibition represents a promising strategy to overcome trastuzumab resistance. A molecular docking study of **80** with the homology model of a HSP90 homodimer showed that **80** fit nicely in the C-terminal domain with a higher electrostatic complementary score than that of ATP.

Human epidermal growth factor receptor 2 (HER2) is amplified in 20-30% of all breast cancers, and amplification of the gene correlates with a poorer prognosis.[1-3] HER2 is a member of the HER receptor tyrosine kinase family, which also includes EGFR (HER1), HER3 and HER4.[4] The interactions between the intracellular domains of the HER receptors lead to the activation of the kinase domains and the subsequent transphosphorylation of several tyrosine residues. The phosphorylation event promotes the expression of genes that act together to regulate cell survival, proliferation and differentiation, which drives tumor progression.[5-7] Trastuzumab, a humanized anti-HER2 monoclonal antibody, has significant clinical benefits for disease-free patients and improves the overall survival of patients with HER2-amplified metastatic and early breast cancer.[8-9] However, nearly 70% of patients do not respond to trastuzumab when given as a single agent therapy due to either primary or acquired resistance, suggesting that overcoming this issue remains a large unmet clinical need.[10]

Heat shock protein 90 (HSP90) is a molecular chaperone that is responsible for the functional competence of HER2 by regulating the stability, maturation and activity of HER2.[11-12] Since HER2 is one of the most sensitive client proteins of HSP90, the inhibition of HSP90 could be an effective strategy for the treatment of HER2-positive breast cancer. HSP90 is a homodimeric protein approximately 90 kDa in size, and each monomer contains an *N*-terminal ATP-binding domain, a middle cochaperone and client-binding domain, and a *C*-terminal dimerization domain. Most HSP90 inhibitors that have been clinically studied were identified to bind to the ATP-binding domain in the *N*-terminus.[13-14] However, although these *N*-terminal inhibitors have shown encouraging efficacy in clinical trials, these inhibitors induce a heat shock response (HSR) that ultimately leads to an increase in the production of HSP90 and anti-apoptotic proteins, such as HSP70 and HSP27. Therefore, the inhibition of a different ATP-binding site in the *C*-terminus provides an alternative strategy to overcome these side effects.[15-18] The extensive efforts directed at the development of the C-terminal inhibitors as well as isoform-selective inhibitors have been reported based on natural products such as novobiocin and deguelin.[19-20]

Deguelin (**1**) is a naturally occurring rotenoid that exhibited potent antitumor activity by inhibiting ATP binding to the C-

term
a C-terminal HSP90 inhibitor based on deguelin, we[22] and other group[23] have investigated simplified deguelin analogues through a ring-truncation approach. The simplified deguelin surrogates displayed more potent antiproliferative and antiangiogenic activities compared to that of deguelin. The mechanistic studies revealed that the ring-truncated analogues also disrupted HSP90 function by binding to its C-terminal ATP-binding pocket and interfering with the interaction with its cochaperones and client proteins, triggering their degradation.[22]

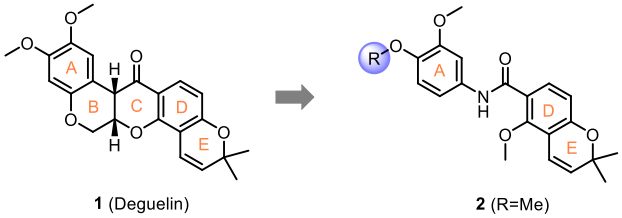
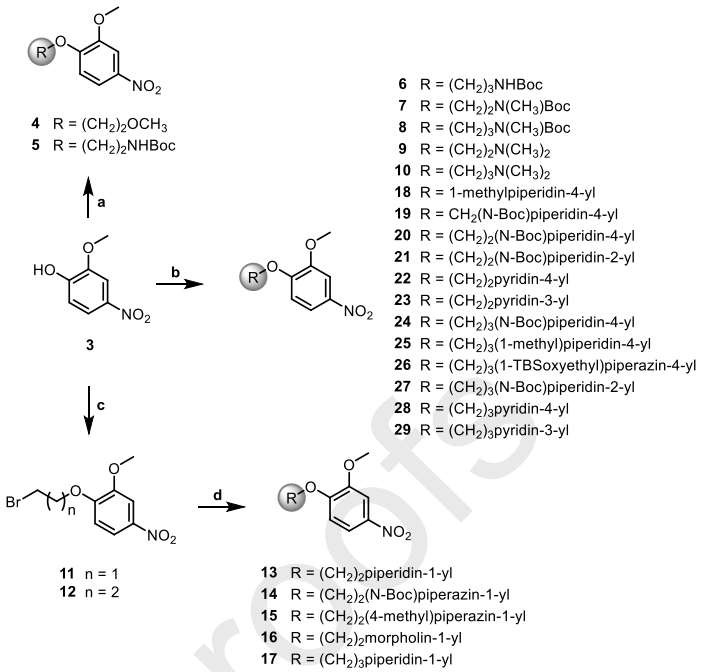


Figure 1. Deguelin and its B,C-ring-truncated analogue

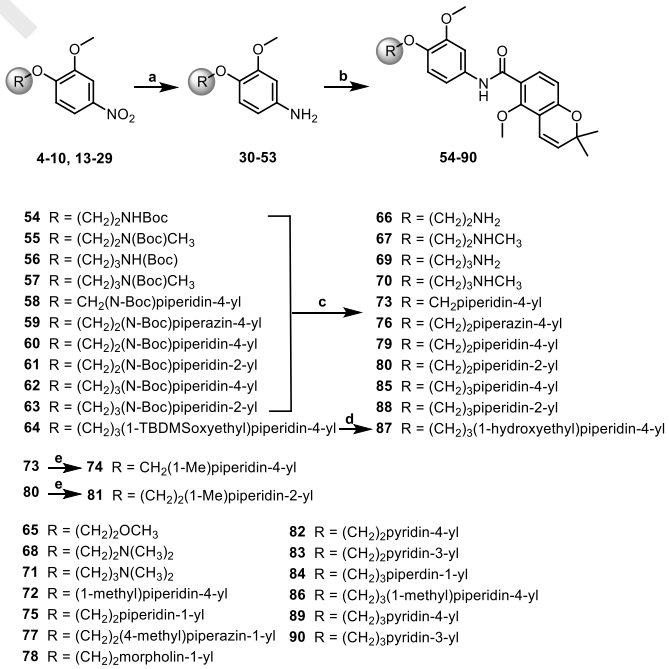
In this study, we have investigated *O*-substituted analogues of the B,C-ring truncated template with an amide linker (e.g., **2** for R=Me) as anti-breast cancer agents in which a variety of polar side chains as solubilizing moieties were incorporated to improve the pharmacodynamic and pharmacokinetic properties of the analogues. The synthesized compounds were screened and evaluated for their cytotoxicity towards two types of HER-2-positive breast cancer cells, trastuzumab-sensitive and trastuzumab-resistant cells, along with normal human cells. Once a potent inhibitor was found in the series, we sought to explore its concentration-dependent cytotoxicity in breast cancer cell lines. In addition, we explored the effects of the inhibitor on the major clients of HSP90 in a mechanistic study and performed molecular modeling to identify the binding mode of the inhibitor in the C-terminus of HSP90.

Generally, the final compounds were synthesized by coupling between the A-ring amines, which were prepared from 4-nitroguaiacol derivatives, and 5-methoxy-2,2-dimethyl-2*H*-chromene-6-carboxylic acid acted as the D-E rings. The acyclic side chains were incorporated into 4-nitroguaiacol by either the Williamson reaction with the corresponding halides to give **4-5** or the Mitsunobu reaction with the corresponding alcohols to provide **6-10**. The cyclic side chains were also incorporated using these two methods. First, 4-nitroguaiacol was alkylated with 1,2-dibromoethane or 1,3-dibromopropane to produce *O*-bromoalkyl derivatives (**11**, **12**), which were then reacted with the corresponding amines to yield **13-17**. Using the Mitsunobu reaction with compound **3** and the corresponding alcohols provided **18-29** (**Scheme 1**).

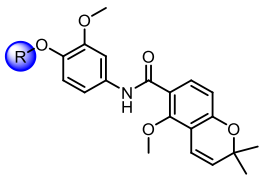
For the synthesis of the final compounds (**Scheme 2**), the 4-nitroguaiacol derivatives (**4-10**, **13-29**) were reduced to their corresponding amines and then coupled with chromene acid under 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) conditions to yield the final compounds (**65**, **68**, **71**, **72**, **75**, **77**, **78**, **82-84**, **86**, **89**, and **90**) or the penultimate products (**54-64**). The Boc or TBS deprotection of **54-64** resulted in the final compounds (**66**, **67**, **69**, **70**, **73**, **76**, **79**, **80**, **85**, **87**, and **88**). The *N*-methyl derivatives **74** and **81** were obtained from **73** and **80** by reductive amination, respectively.



Scheme 1. *Reagents and conditions:* (a) RBr or RI, K_2CO_3, DMF, 70 °C, 3 h; (b) ROH, PPh_3, DEAD, CH_2Cl_2, 0 °C-r.t., overnight; (c) $BrCH_2(CH_2)_nBr$, K_2CO_3, DMF, 70 °C, 3 h; (d) amine (piperidine, morpholine, 1-methylpiperazine, or *N*-Boc piperazine), DMF, 70 °C, overnight



Scheme 2. *Reagents and conditions*: (a) H_2, Pd/C, MeOH, 2 h; (b) 5-methoxy-2,2-dimethyl-2*H*-chromene-6-carboxylic acid, EDC.HCl, HOBt, TEA, CH_2Cl_2, reflux, overnight.; (c) TFA, CH_2Cl_2, r.t., overnight; (d) TBAF, CH_2Cl_2, r.t., overnight; (e) (HCHO)n, $ZnCl_2$, CH_2Cl_2, r.t., 1 h; then $NaBH_4$, reflux, overnight



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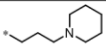
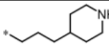
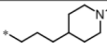
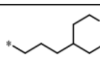
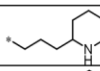
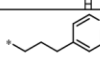
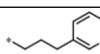
66		75		84	
67		76		85	
68		77		86	
69		78		87	
70		79		88	
71		80		89	
72		81		90	

To examine the primary antiproliferative activities of the synthesized compounds (**Table 1**) in HER2-positive breast cancer cells, we evaluated the cytotoxicity of the 27 compounds in trastuzumab-sensitive BT474 cells, trastuzumab-resistant JIMT-1 cells (**Figure 2**) and normal human embryonic kidney (HEK293) cells at a concentration of 10 μM for 72 h. Among the tested compounds, nine compounds (**73**, **74**, **80**, **81**, **84**, **85**, **86**, **87**, and **88**) suppressed cell viability by levels below 50% in both the BT474 and JIMT-1 cells (**Figure 2A and 2B)**. In particular, compound **80** exhibited significant inhibition of cell proliferation in HER2-positive breast cancer cells, while it had minimal cytotoxicity in normal HEK293 cells (**Figure 2C)**.

Among the compounds tested in the primary screening, compound **80** showed the most promising cytotoxicity in HER2-positive breast cancer cells**.** To further examine the concentration-dependent cytotoxicity of **80**, BT474 and JIMT-1 cells were treated with various concentrations of **80** (0.1, 0.5, 1, 5, 10 or 20 μM) for 72 h. MTS assays revealed that **80** (1-20 μM) significantly suppressed the cell viability in both BT474 and JIMT-1 cells in a dose-dependent manner. The inhibitory concentrations (IC_{50}) of **80** in BT474 (a) and JIMT1 (b) cells were 8.53 μM (95% CI: 6.429 - 11.33 μM)] and 4.45 μM (95% CI: 2.959 - 6.691 μM), respectively (**Figure 3).**

We examined the effect of **80** on the expression and activation of the major clients of HSP90, including HER2, EGFR and survivin, in BT474 and JIMT-1 cells *in vitro*. Treatment with **80** (IC50 values of approximately 5-10 μM) markedly downregulated the expression of the total HER2 and total EGFR proteins, concomitant with decreases in their phosphorylation in both trastuzumab-sensitive and trastuzumab-resistant cells (**Figure 4A**), suggesting that HSP90 inhibition represents a potential strategy to overcome trastuzumab resistance.

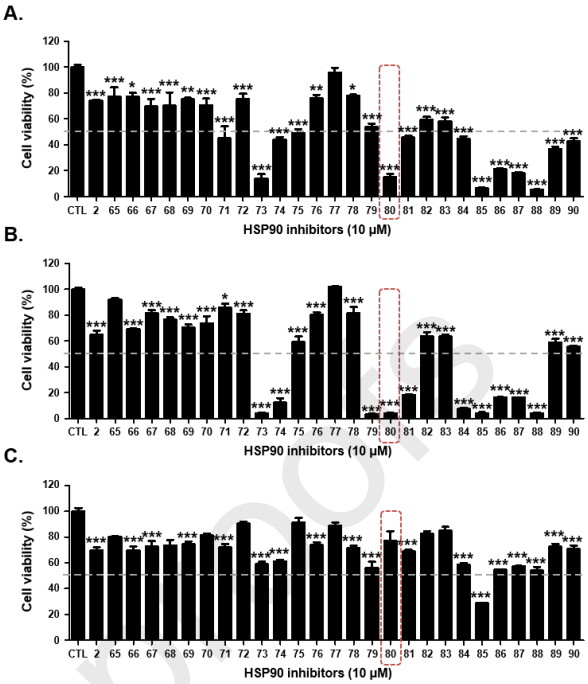


Figure 2. Primary screening of NCT analogues (2, 65-90) (a-c) Effects of the synthesized 27 compounds on cell viability in HER2-positive breast cancer cells and normal human embryonic kidney HEK293 cells. Trastuzumab-sensitive BT474 (a), trastuzumab-resistant JIMT-1 (b) and normal HEK293 (c) cells were treated with each of the compounds at 10 μM for 72 h, and the cell viability was assessed using a MTS assay. The red dotted boxes indicate the antiproliferative activity of compound **80** in cancer cells (a-b) and normal cells (c). The results are presented as the mean ± SEM of at least three independent experiments and analyzed using Student's t-test (* p<0.05, versus DMSO control).

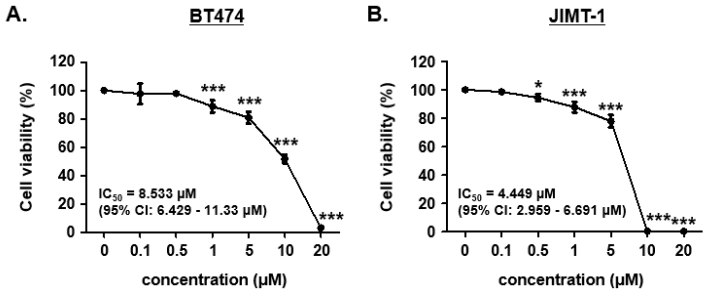


Figure 3. Effect of compound of 80 on cell viability in trastuzumab-sensitive and trastuzumab-resistant HER2-positive breast cancer cells. (a-b) Effect of **80** on the cell viability in BT474 (a) and JIMT-1 (b) cells. The cells were treated with various concentrations of **80** (0.1, 0.5, 1, 5, 10 or 20 μM) for and 72 h. The cell viability was determined by MTS assay. The results are presented as the mean ± SEM of at least three independent experiments and analyzed by one-way ANOVA followed by Bonferroni's *post hoc* test (* p<0.05, versus DMSO control). IC_{50} values and 95% confidence intervals (CI) were calculated with GraphPad software (version 5; GraphPad Prism, San Diego, CA).

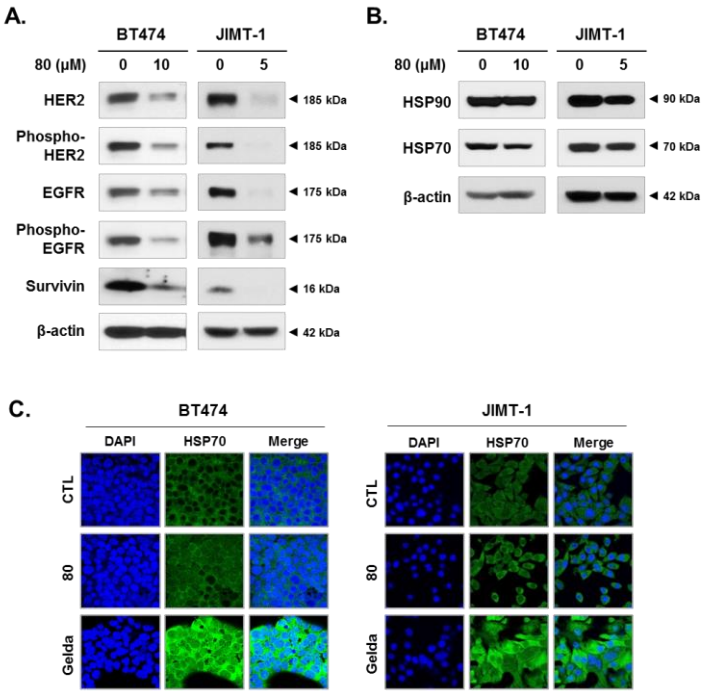


Figure 4. Effect of compound 80 on the expression of HSP90 client proteins and HSP70 in HER2-positive breast cancer cells. (a) BT474 and JIMT-1 cells were treated with the indicated concentrations of 80 for 72 h. Equal amounts (30 μg) of protein from the total cell lysates were separated by SDS-PAGE and immunoblotted using HER2, phospho-HER2 (Tyr1221/1222), EGFR and phospho-EGFR (Tyr1068), survivin antibodies. Actin was used as a loading control for the total cell lysates. (b) Immunoblot analyses of HSP90 and HSP70 protein expression in BT474 and JIMT-1 cells following exposure to 80 for 72 h. (c) Representative confocal images of immunocytochemistry for HSP70 expression. Cells were immunostained for HSP70 (green) and nuclei were counter-stained with DAPI (blue) after treatment of geldanamycin (Gelda; 0.3 μM) or 80 (10 μM) for 24 h. Microphotographs were taken using confocal microscopy (original magnification: ×500).

The anti-apoptotic protein survivin, a member of the inhibitor of apoptosis protein (IAP) family, is an important determinant in the regulation of cell division and proliferation and in the evasion of apoptosis.[24,25] Exposure to **80** considerably decreased the survivin protein content in both BT474 and JIMT-1 cells (**Figure 4A**). We next examined whether 80 treatment is associated with the induction of HSR in HER2-positive breast cancer cells, expression levels of HSP90 and HSP70 in the presence or absence of **80** were determined by Western blot analysis. There were no differences in protein expression of HSP90 and HSP70 between the control vehicle- and 80-treated cells (**Figure 4B**). To confirm this observation, immunocytochemical analysis for HSP70 in the presence of 80 or N-terminal HSP90 inhibitor, geldanamycin was performed. Further evidence supported the notion that exposure to 80 did not affect HSP70 expression, while N-terminal HSP90 inhibitor, geldanamycin treatment resulted in a marked upregulation of HSP70 (**Figure 4C**). These observations suggest that the compound **80**-induced inhibitory effect on cancer cell proliferation could be caused by the destabilization and inactivation of HSP90 client proteins without the induction of HSR.

cted to determine the binding mode of compound **80** in the C-terminal domain of HSP90. The flexible docking of **80** was analyzed using the homology model of the previously prepared open form of a HSP90 homodimer.[26,27] As illustrated in **Figure 5A**, compound **80** fits well in the C-terminal domain, and the 2-piperidine moiety occupies the ATP binding pocket where the deoxyribose moiety is located. The protonated amine group forms a strong hydrogen bond with the carboxy side chain of Glu611. Two aromatic groups, from the central benzene and chromene rings, create π-cation interactions with Lys615 of each HSP90 chain. The methoxy and amide carbonyl oxygen groups of compound **80** also form hydrogen bonds with Asn622 and Lys615 (A), respectively. These various interactions allow compound **80** to be in contact with both chains of HSP90 and stabilize the open form of the HSP90 homodimer (**Figure 5B**). We also conducted docking analysis for both the *R* and *S* form of compound **80** and obtained similar poses and docking scores, suggesting that stereochemistry is only a minor factor in the compound's activity (data not shown). Last, we explored the electrostatic complementarity (EC) surface and score of compound **80** by using the Flare program.[28] By using XED force field calculations, the complementarity of the ligand and protein were visualized. As shown in **Figure 5C**, the overall structure of compound **80** is electrostatically favored by HSP90. Both the electrostatic complementarity (EC) surface and score indicate that compound **80** binds well to HSP90, and since compound **80** has a higher EC score than ATP, these results support the idea that compound **80** is an effective C-terminal HSP90 inhibitor.

In summary, to discover C-terminal terminus inhibitor of HSP90 as novel anti-breast cancer agents, we investigated a series of *O*-substituted analogues of the B,C-ring truncated template of deguelin with an amide linker as anti-breast cancer agents in which a variety of polar side chain were incorporated. The synthesized compounds were evaluated for their cytotoxicities towards two types of HER2-positive breast cancer cells, trastuzumab-sensitive (BT4747) and trastuzumab-resistant (JIMT-1) cells, and normal human cells in the primary screening. Among the synthesized compounds, compound **80** exhibited significant inhibition of cell proliferation in HER2-positive breast cancer cells, with an IC_{50} = 8.53 and 4.45 μM in BT474 and JIMT1, respectively, whereas compound **80** exhibited minimal cytotoxicity in normal HEK293 cells. A mechanistic study of **80** indicated that it markedly downregulated the expression of the total HER2 and total EGFR proteins, concomitant with decreasing their phosphorylation, and additionally considerably decreased the content of the anti-apoptotic protein survivin in both BT474 and JIMT-1 cells. The immunocytochemical analysis for HSP70 in the presence of **80** or N-terminal HSP90 inhibitor indicated that the inhibitory effect of **80** on cancer cell proliferation was caused by the destabilization and inactivation of HSP90 client proteins without the induction of HSR as evidence of the C-termial inhibitor. These results indicate that the cytotoxicity of compound **80** in breast cancer cells is attributed to the destabilization and inactivation of HSP90 client proteins and that HSP90 inhibition is a promising strategy to overcome trastuzumab resistance. A molecular docking study of compound **80** with the homology model of the open form of the HSP90 homodimer indicated that compound **80** fit well in the C-terminal domain through key interactions with the domain, and the electrostatic complementary score of compound **80** was higher than that of ATP.

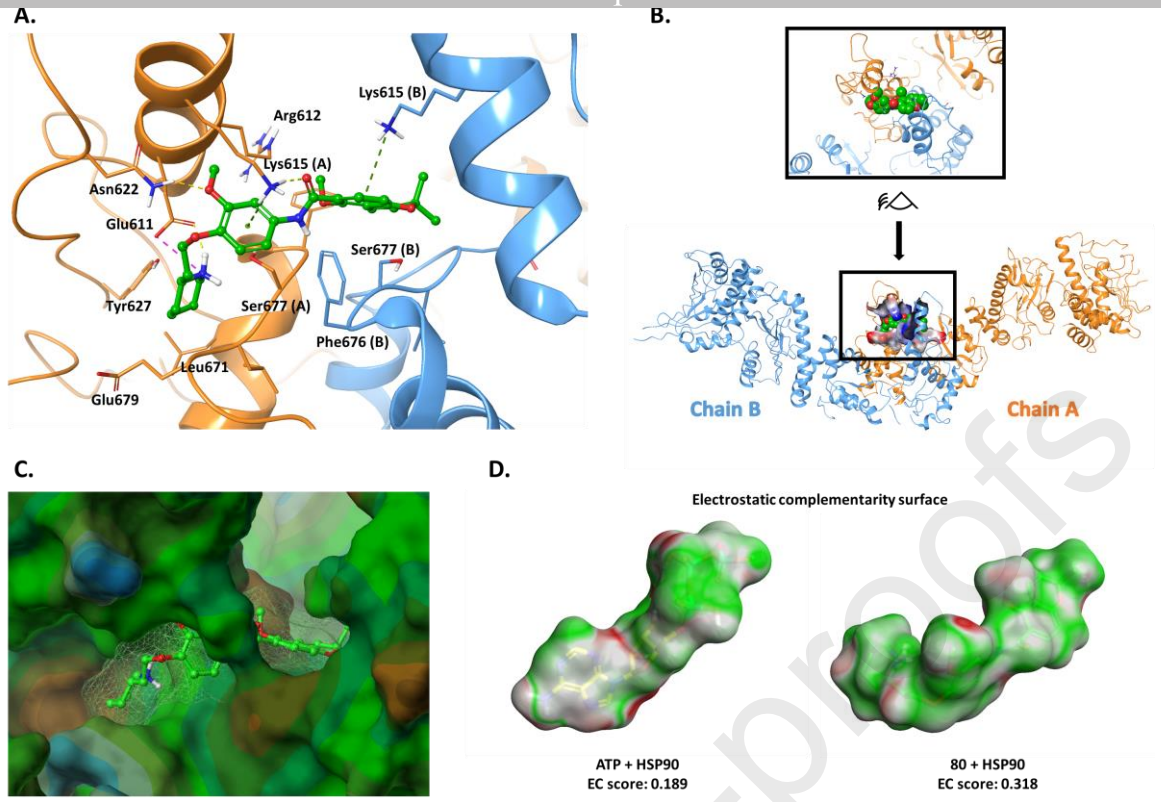


Figure 5. Molecular docking model of compound 80 with HSP90. (A) Compound **80** represented as a green ball-and-stick model (Surflex-Dock score: 8.3236, CScore: 5). The two HSP90 chains are indicated in orange (Chain A) and sky-blue (Chain B). The hydrogen bonds, π-cation interactions and salt bridges are represented as yellow, green and magenta dashed lines, respectively. (B) The binding site of **80** in the dimerization interface. Overall, the HSP90 homodimer is represented as a ribbon, and the binding site is represented with an electrostatic potential surface. The top view of the **80** docking model is highlighted in the upper black box. (C) Lipophilic potential surface (brown color: hydrophobic; blue color: hydrophilic) of the HSP90 homodimer and **80**. The Connolly surface of **80** is represented by a green mesh. (D) The electrostatic complementarity (EC) surface and score. Green = perfect electrostatic complementarity, red = perfect electrostatic clash.

Acknowledgments

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Graphical Abstract

Discovery of Novel Anti-Breast Cancer Agents Derived from Deguelin as Inhibitors of Heat Shock Protein 90 (HSP90)

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