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Structure-based discovery of a specific TLR1-TLR2 small molecule agonist from ZINC drug library database

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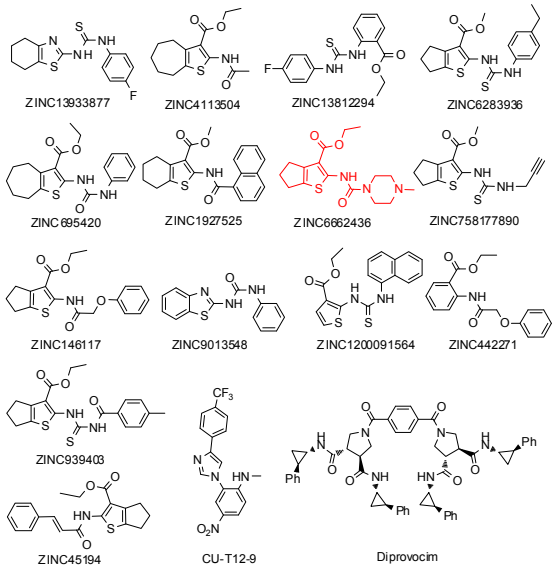
We report herein the identification of the urea structure-like small molecules from structure-based virtual screening of 10.5 million compounds. Base on variety of HEK-Blue hTLRs reporter cells assay results, we validated a TLR1/2-specific small molecule agonist ZINC666243 (SMU127) with EC_{50} of 0.55±0.01 μM. SMU127 stimulates the NF-κB activation and promotes TNFα secretion in human macrophage and mononuclear cells. Moreover, the in vivo assay indicated that SMU127 could inhibit the breast cancer tumors growth in BABL/c mice. This work has shown for the first time that the small molecule TLR1/2 agonist can inhibit breast cancer *in vivo*.

The ability of toll-like receptors (TLRs) to activate both immune systems has made them desirable targets both for vaccine adjuvants and/or cancer treatment. For example, the modified TLR4 agonist, monophosphoryl lipid A (MPLA), is used as an adjuvant in the FDA approved adult cervical cancer vaccine.[1] Imiquimod (INN) is a FDA approved drug which functions through TLR7 and used for the treatment of genital warts, superficial basal cell carcinoma and actinic keratosis.[2] Other TLR agonists are also being investigated for vaccine adjuvant uses including the TLR9 agonist CpG-ODN in HBV vaccines, TLR2 agonist (rLP208-A05 and rLP2086-B01) in the FDA-approved Trumenba vaccine.[3]

Recently, a TLR2 agonist, polysaccharide krestin (PSK), extracted from mushroom is being tested in a clinical phase II trial now for the treatment of breast cancer.[4] Accumulated evidences have proven that TLR2 agonists could be used directly,[5, 6] or as structurally modified forms[7] in cancer treatment, suggesting that TLR2 agonists can be potential effective enhancers for cancer immunotherapies. Administration of TLR2 agonists could enhance effector and memory T cell responses, leading to elevate efficacy of vaccination and tumor rejection.[8, 9] During the submission of our manuscript, Beutler and Boger discovered a new TLR1/2 activator, Diprovocim, which was 100% cured against mouse melanoma in combination with the PD-L1 antibody.[10] These potential applications and exciting results motivated us to discover and develop novel TLR2-selective agonists. This task is particularly challenging, for TLR2 recognizes a wide range of ligands, many of which are from Gram-positive bacteria, and it signals as a heterodimer with either TLR1 or TLR6,[11] raising questions about the specificity of small molecule agents. Second, until today, none of the TLR2 agonists in clinical or preclinical development are small molecules, and some contain mixtures with more than one active ingredient. Our group has focused on small molecules that target the TLR2 heterodimer protein-protein interaction,[12, 13] specifically modulating the TLR1/2 response.[14-16] In this study we aim to discover new TLR1/2 small molecule modulating agents through computer-aided drug design which we have successfully employed in our previous research.[10] In search of small molecule probes, the 10,519,614 compounds from



Scheme. 1 Chemical structure of the 14 top hits, Diprovocim[10] and CU-T12-9[13].

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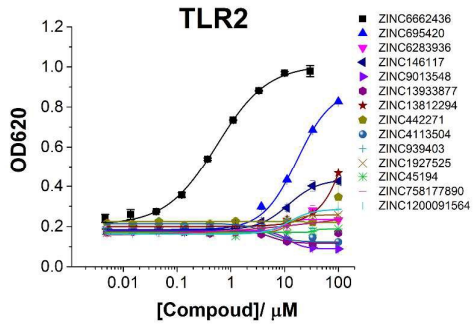

Fig. 1. Comparison of the initial hits in the TLR2 related secreted alkaline phosphatase (SEAP) gene activation. HEK-Blue hTLR2 cells were incubated with indicated concentrations (0-100μM) of the initial hits for 24 hours, and the activation was measured using Quanti-Blue reagent by determining alkaline phosphatase secretion in the culture supernatants at A620. Data presented are mean ± SD and the figures shown are representative of three independent experiments.

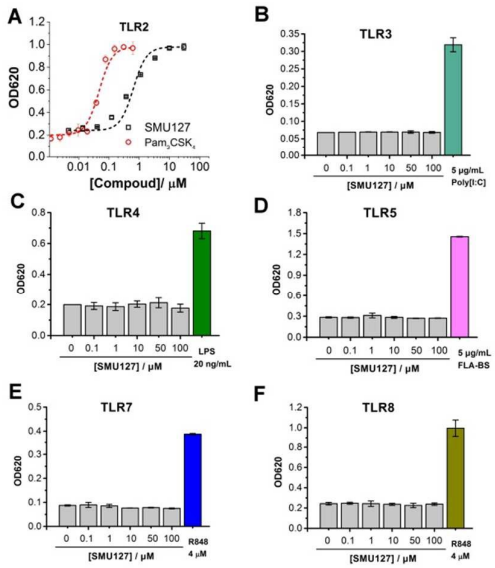

Fig. 2. Specificity test for SMU127 in different HEK-Blue hTLRs. (A) SMU127 stimulates signaling of SEAP in a dose-dependent manner. (B to F) HEK-Blue Human TLR3 (B), TLR4 (C), TLR5 (D), TLR7 (E), and TLR8 (F) cells were incubated with SMU127 (0 to 100 μM) with the control of TLR-specific agonists separately for 24 hours, and activation was evaluated by the optical density at 620nm. Agonist: TLR3, poly(I:C) (5 μg/ml); TLR4, LPS (10 ng/ml); TLR5, FLA-BS (5 μg/ml); TLR7 and TLR8, R848 (4 μg/ml). Data presented are mean ± SD and the figures shown are representative of three independent experiments.

ZINC database was screened against the Pam_3CSK_4-binding domain of TLR1/2 (crystal structure PDB: 2Z7X)[11] using the Glide 7.4 program (Fig. S1 and S2). Base on the computer scoring results, including shape, chemical-feature, drug-like properties, 14 compounds were finally selected out and purchased from ZINC (Scheme 1), and then verified their activity *in vitro*. Interestingly, almost all of the hits identified, with ZINC6662436 as a representative structure, from the *in silico* screening generally share the common motif of an amine conjugated with an acid (isocyanic/isothiocyanic/carboxylic) substituent, implying a novel pharmacophore to target the Pam_3CSK_4-binding site of TLR1/2.

These initial hits were first evaluated using our previously established cell assay of TLR2 activation. HEK-Blue hTLR2 cells were employed to selectively activate TLR2 signaling, through the adaptor proteins MyD88/TRAF/IKK NF-κB pathway to active the NF-κB signaling. This triggers the secreted alkaline phosphatase (SEAP) promoter to secrete alkaline phosphatase, or induces production of pro-inflammatory cytokines. We monitored the SEAP level as an indicator of small molecule-induced TLR2 activation to evaluate the compound's activity (Fig. 1). Fortunately, two of fourteen compounds demonstrated relatively higher TLR2 activation effects in the whole cell assay (Scheme 1 and Fig. 1), with ZINC6662436 (SMU127) being particularly excellent (EC_{50} at 0.55± 0.02 μM). Therefore, SMU127 was selected out and synthesized (Scheme S1, Fig. S3-S6) for further target validation and potency evaluation.

To test whether the SMU127 can specifically activate the TLR2 signaling pathway, HEK-Blue hTLRs cells transfected with SEAP reporter genes were used, including hTLR3, 4, 7 and 8. Cell culture supernatant was collected to test the induction of NF-κB signaling pathway. The representative dose-dependent curve showed SMU127 had similar activation mode as the well-studied TLR1/2 agonist Pam_3CSK_4. As shown in Figure 2B to 2F, SMU127 specifically activates TLR2 rather than other TLRs, including TLR3, TLR4, TLR5, TLR7 and 8. Next, we tested whether SMU127 binds to TLR2 on the cell surface and induces TLR2 expression. HEK-Blue hTLR2 cells, which overexpress the human TLR2, were stimulated with SMU127. Cell lysates were subjected to immunoprecipitation and immuno-detected using TLR2 antibody. As shown in the Figure 3A, as the dose of SMU127 increased, the expression of TLR2 was gradually increased, showing that SMU127 interacts with TLR2 in a whole-cell environment. HEK-Blue hTLR2 cells can endogenously express TLR1 and TLR6, therefore whether SMU127 is an activator of TLR1/2 or TLR2/6 still remains unclear. Here, we performed an antibody inhibition experiment to test its selectivity. SMU127 can efficiently activate SEAP signaling at the concentration of 0.5 μM, and addition of anti-hTLR1 and anti-hTLR2 but not anti-hTLR6 antibodies changed such activation in a dose-dependent way (Fig. 3B). To confirm whether the anti-hTLR6 antibodies are functional, we used a TLR2/6 agonist Pam_2CSK_4, and tested the SEAP activation in the same cell line, and found that both anti-hTLR2 and anti-hTLR6 antibodies can restrain Pam_2CSK_4-induced SEAP signaling, whereas anti-hTLR1 antibodies did not block the activity (Fig. 3C). Moreover, one oxygen and two nitrogen form urea skeleton of SMU127 have very distinct hydrogen bonding with the key amino acid residues Phe312, Gln316 and Gly313 in TLR1[11], and hydrophobic interaction between cyclopentathiophene ring and Phe349 in TLR2 also

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been observed (Fig. 3C). These observations further confirmed that SMU127 acts as an agonist of TLR1/2.

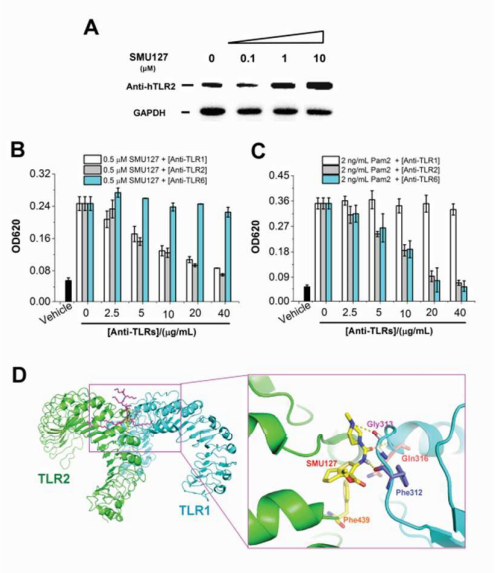

Fig. 3. SMU127 was proved to be specific ligand of TLR1/2, not TLR2/6. (A) SMU127 promoted the TLR2 protein expression in HEK-Blue hTLR2 cells. (B) HEK-Blue hTLR2 cells were treated with SMU127 and antibodies of anti-hTLR1, anti-hTLR2, or anti-hTLR6 for 24 hours. Anti-hTLR1-IgG and anti-hTLR2-IgA antibodies can inhibit SMU127-triggered SEAP signaling on a dose-dependently manner, whereas anti-hTLR6-IgG has no influence to the SEAP signaling. (C) The positive control of Pam_2CSK_4, a TLR2/6 agonist, showed different responses to TLR1, 2 and 6 antibodies comparing with SMU127. Data are means ± SD of triplicate and representative of three independent experiments. (D) Low-energy binding conformations of SMU127 and Pam_3CSK_4 bound to the TLR1–TLR2 interface as generated by virtual ligand docking.

In order to investigate the cellular and molecular mechanisms of SMU127 with NF-κB, we developed a TLR2-sensitive U937 human macrophage cell line with a GFP-labeled NF-κB reporter. Flow cytometry experiments demonstrated that SMU127 activated NF-κB signaling in a dose-dependent manner. At the dose of 1 μM, SMU127 showed comparable activation to 100 ng/ml of Pam_3CSK_4, and at a higher dose (10 μM) even had a better effect (Fig. 4A-4F). To investigate the cell signaling of SMU127 induced activation, we employed a known NF-κB inhibitor, triptolide. Triptolide was able to efficiently inhibit the SMU127 induced SEAP signaling (Fig. 4G), which further implies that SMU127 works through the NF-κB signaling pathway. A key output from this activation is tumor necrosis factor-alpha (TNF-α), which is proven to be directly relevant to inflammatory diseases and cancer. To ascertain whether the NF-κB activity induced by SMU127 reflected upregulation of TNF-α, we assessed the ability of SMU127 to activate TNF-α in human PBMC using an ELISA experiment. The experiment showed SMU127 possesses the ability to activate TNF-α signaling in a dose-dependent manner (Fig.4H).

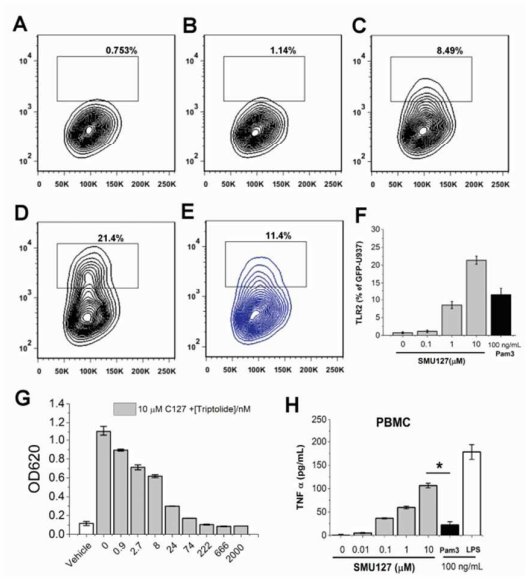

Fig. 4. SMU127 works through NF-κB pathway to activate the downstream signaling. Representative flow graphs of the TLR2-sensitive U937 human macrophage cell line with a GFP-labeled NF-κB reporter treated with (A) 0, (B) 0.1, (C) 1, (D) 10, and (E) 100 ng/mL Pam_3CSK_4 for 24 hours before determination by flow cytometry. (F) Summarized the results from A to E, and the result demonstrated SMU127 had excellent NF-κB activation compared with the positive control Pam_3CSK_4. (G) The NF-κB activation of SMU127 can be inhibited by a NF-κB inhibitor, triptolide, in HEK-Blue hTLR2 cells. (H) SMU127 triggers TNF-α production after the TLR1/2 activation in human peripheral blood mononuclear cells (PBMC). Data are means ± SD of triplicate and representative of three independent experiments. *P<0.01.

Previous reports suggest that TLR1/2 agonist BLP (bacterial lipoprotein) can effectively inhibit lung cancer, leukemia, melanoma, and lead to a long-lasting protective response against tumor rechallenge.[17] Moreover, TLR1/2's agonist polysaccharide krestin (PSK) has shown amazing effects in the inhibition of both implanted and spontaneous breast tumors and is already in phase II clinical trial.[4] SMU127 has a good TLR1/2 activation effect at the cellular level, and does it also have antitumor activity in vivo? In this report, we evaluated its anti-breast cancer activity in the BABL/c mice model. The results indicated that SMU127 decreased the progression speed of breast cancer volume by compared with PBS as blank control in BABL/c mice. Generally, groups of female BABL/c mice were implanted with syngeneic 4T1 breast cancer cells (3×10^5 cells/mice, ~100 μl in PBS); on day 7 when tumors were palpable, mice were treated with intraperitoneal injection of SMU127 (0.1 mg per mice) or PBS and repeated every two days. Tumor size and survival in these mice were monitored regularly. As shown in Figure 5, SMU127 treatment significantly inhibits the growth of breast tumor in BABL/c mice. The tumor size after 24 days of treatment was 1035±196 mm^3 in the SMU127 group and 2256±150 mm^3 in the PBS group. In addition, treatment with SMU127 significantly

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reduced the tumor volume and weight (Figure 5A, 5C, 5D), but no reduction in body weight as compared with the control (Figure 5B). Although SMU127, as the first TLR1/2 small molecule ligand that inhibits breast cancer *in vivo*, there is still a clear room for further efficacy improvement. In view of the synergistic anti-melanoma effect of TLR1/2 activator Diprovocim and PD-L1 antibody,[10] combined SMU127 with PD-L1 antibody or other immunological checkpoint inhibitors may be a new direction to improve the tumor immunity. During the tumor immunization, IL-10 needs to be considered for it will influence the TLR2 ligands anti-tumor effect.[18,19] These ideas and indicators will be confirmed in our future research.

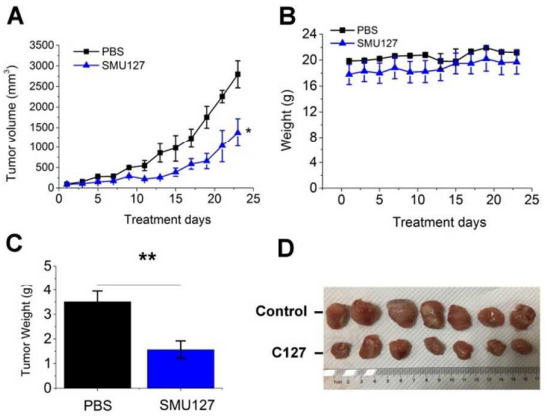

Fig. 5. SMU127 inhibits the growth of implanted 4T1 breast cancer cells in BABL/C mice. Female BABL/c mice were inoculated with 4T1 cells (3×10^5 cells/mice) for 7 days and the solid tumors established, then the mice were treated intraperitoneally with PBS, SMU127 (0.1 mg, i.p.) once every two days. The tumor volumes and body weights of individual groups of mice were monitored. Data are expressed as the mean ± SD of individual groups of mice (n = 7 per group). (A) The volumes of tumors. (B) The body weights of mice. (C) The weight of tumors. (D) Image of the tumors. *P < 0.01 vs control at the last measurement, **P < 0.001.

In conclusion, we have identified compound SMU127 as a potential immunomodulatory small molecule agonist of TLR1 and TLR2 by structure-based virtual screening. The stimulating effect in different HEK-TLR cells assays proved its specificity. Next, an immunoprecipitation assay demonstrated that SMU127 promoted the TLR2 protein expression in HEK-Blue hTLR2 cells. Further antibodies experiments confirmed SMU127 activated TLR1/2, not TLR2/6. Then, SMU127 worked through NF-κB signalling pathway and promoted the release of TNF-α in human peripheral blood mononuclear cells. What's more, SMU127 showed significantly tumor immune effect to against breast cancer *in vivo*. To our knowledge, SMU127 is the second TLR1/2 small molecule agonist which works *in vivo* and only the third small molecule scaffold agonist overall of TLR1–TLR2 heterodimerization reported in the literature. Taken together, our work enriched the situation of lacking of small-molecule of TLR1/2 agonist family, and *in vitro*/*vivo* assays explores the working mechanism of SMU127 and provides the potential therapeutic applications in vaccine adjuvants and tumor immunity therapies.

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Conflicts of interest

There are no conflicts to declare.

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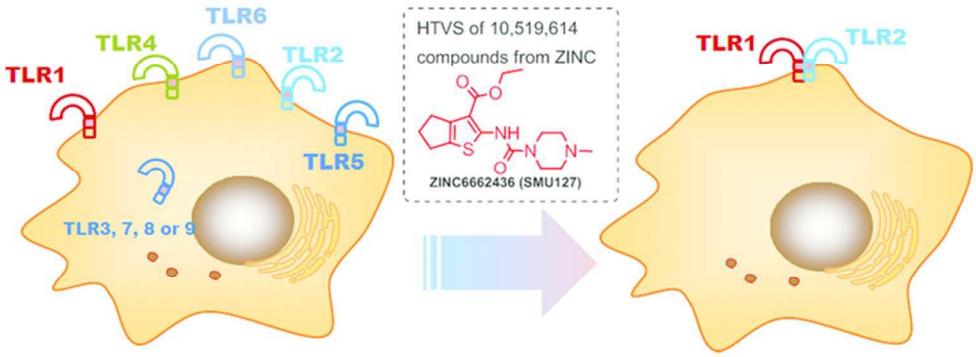

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