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Novel non-peptidic small molecule inhibitors of secreted aspartic protease 2 (SAP2) for the treatment of resistant fungal infections†

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Targeting secreted aspartic protease 2 (SAP2), a kind of virulence factor, represents a new strategy for antifungal drug discovery. In this report, the first-generation of small molecule SAP2 inhibitors was rationally designed and optimized using a structure-based approach. In particular, inhibitor 23h was highly potent and selective and showed good antifungal potency for the treatment of resistant *Candida albicans* infections.

Invasive fungal infections (IFIs) represent a serious health risk with high incidence and mortality in immunocompromised individuals.[1] Despite the availability of several classes of antifungal agents (*e.g.* polyenes, triazoles and echinocandins) for IFI treatment, the mortality is still on the rise due to limited clinical efficacy, drug-related toxicity, and severe drug resistance.[2] Thus, there is an urgent need to validate new antifungal drug targets as well as developing a new generation of safe and effective antifungal agents.[3]

In recent years, targeting virulence factors has represented a new therapeutic approach in antimicrobial therapy.[4] Most of the success in this field was focused on the discovery of new drugs targeting bacterial virulence.[5–7] In contrast, effective small molecules targeting fungal virulence factors are very rare.[8] Secreted aspartic proteases (SAPs) of *Candida albicans* are a family of important virulence factors for localized and systemic infections.[9] In *Candida albicans*, ten distinct SAP genes (SAP1-10) were expressed *in vitro* and *in vivo*.[10] The SAPs are essential for the fungal nutrition process and contribute to the fungal pathogenicity due to their critical participation in several stages of the infective process including adhesion, invasion, tissue damage and so on.[9,11] Moreover, a higher secretion of SAP was observed in drug-resistant *Candida* spp. isolates than in the susceptible isolates.[9] Thus, the SAPs offer potential targets for the discovery of novel antifungal agents.[12] Moreover, targeting SAPs may provide a novel intervention strategy to overcome drug resistance. Among the SAPs, SAP2 is one of the most expressed enzymes implicated in host invasion and attracted interest in early stages of drug discovery.[9]

Several natural products showed weak or moderate inhibitory activity against the extracts of SAPs.[13] For SAP2, only peptide (*e.g.* pepstatin A, **1**, Fig. 1A)[14] and peptidomimetic[15,16]

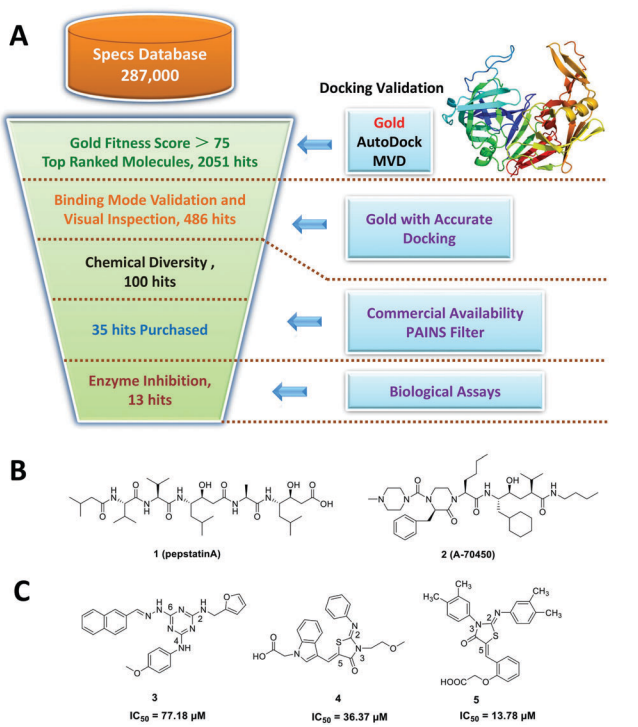

Fig. 1 Discovery of small molecule SAP2 inhibitors by virtual screening. (A) Protocol for virtual screening of small-molecule SAP2 inhibitors; (B) chemical structure of peptidic SAP2 inhibitors; (C) chemical structures and inhibitory activity of three novel SAP2 inhibitors.

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SAP2 inhibitors were reported. However, it is very difficult to develop peptidic or peptidomimetic compounds into clinical drugs due to significant challenges in achieving suitable pharmacokinetic characteristics. To date, only one report has demonstrated antifungal activity of peptidomimetic SAP2 inhibitors.[15] Thus, it is highly desirable to discover non-peptidic (or non-peptidomimetic) small molecule inhibitors. Such inhibitors are expected to possess both good SAP2 inhibitory activity and good *in vivo* antifungal potency. The crystal structure of SAP2 in complex with the peptidic inhibitor A70450 (**2**, Fig. 1B) has been solved,[17] offering the opportunity of structure-based discovery of small molecule inhibitors. However, therapeutically targeting SAP-like proteases by a small molecule is a highly challenging task.[18] The difficulty lies in how to effectively mimic the interactions of large peptidic inhibitors and achieve a balance of binding affinity and drug-like properties. Herein, we report structure-based discovery of the first generation of small molecule SAP2 inhibitors by combining docking-based virtual screening and structure-based drug design.

Following our established virtual screening protocols (Fig. 1A and Table S1 in the ESI†),[19] 35 compounds (Fig. S1 in the ESI†) were selected from about 287 000 compounds in the Specs 3D database by docking, scoring, visual inspection and Pan-assay interference compound (PAINS, http://zinc15.docking.org/patterns/home) filtration.[20] The evaluation of the *C. albicans* SAP2 inhibitory activity was carried out *via* a spectrophotometric assay.[21] Initial screening was performed at the concentration of 100 μM and 13 out of 35 compounds showed an inhibitory rate larger than 40% (Fig. 1B and Table S2 in the ESI†). The IC_{50} values were further determined for 7 compounds with the inhibitory rate larger than 50%. The results indicated that all of them had an IC_{50} value lower than 100 μM. Among them, three compounds, namely **3** (IC_{50} = 77.18 μM), **4** (IC_{50} = 36.37 μM) and **5** (IC_{50} = 13.78 μM), showed the best SAP2 inhibitory activity (Fig. 1C).

In order to get the binding mode of these novel SAP2 inhibitors, accurate molecular docking was performed. As shown in Fig. 2, the small molecule inhibitors bound with SAP2 by mimicking the interaction of **2** (see Fig. S2 in the ESI† for schematic representation of the interactions). The analysis of the binding mode of these SAP2 inhibitors revealed that they share three common structural features: (1) a heterocyclic scaffold located at the central part of the active site; (2) three side chains attached on the scaffold to form hydrophobic and π–π interactions with the active site; (3) polar functional groups in the side chain as hydrogen bond donors or acceptors. Among the six pockets in the SAP2 active site, S1, S2, S1′ and S2′ are important for small molecule binding.

Due to the importance of selectivity in the discovery of antifungal agents, the three SAP2 inhibitors were assayed for inhibitory activity of beta-secretase 1 (BACE1, a kind of human aspartic protease). The results indicated that compounds **3**, **4** and **5** were inactive at the concentration of 100 μM (inhibition rate <10%). Furthermore, we tested their *in vitro* antifungal activity against *C. albicans* using the standard NCCLS protocols.[22] Interestingly, all of them were inactive in the antifungal assay with minimal inhibitory concentration (MIC) values larger than 64 μg mL^{-1}. The results were well consistent with the action mode of virulence inhibitors.

Inspired by these results, inhibitor **5** was selected for structure–activity relationship (SAR) and structural optimization studies (Fig. 3). First, it was subjected for chemical similarity search in the SPECS database. Seventeen analogues were selected and purchased for biological evaluation (**B1–B17**, Fig. S3 in the ESI†). Among them, compound **6** showed the best activity (IC_{50} = 10.18 μM), which was slightly better than the initial hit **5** (Table S3 in the ESI†). Second, various substituents were introduced onto A-ring and B-ring (compounds **12a–t**, Table S4 in the ESI†) and the importance of the A, B, C-ring

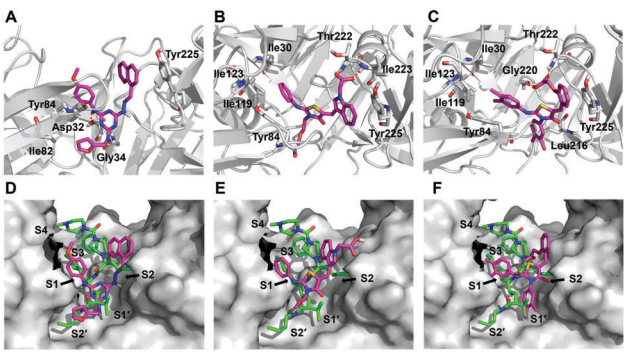

Fig. 2 Proposed binding poses of inhibitors **3** (A), **4** (B) and **5** (C) in the active site of *C. albicans* SAP2. The comparison of binding modes of small-molecule inhibitors (carbon atoms in purple) **3** (D), **4** (E) and **5** (F) with the peptide inhibitor compound **2** (carbon atoms in green) is shown on the surface of SAP2. The figures are generated using PyMol (http://www.pymol.org/).

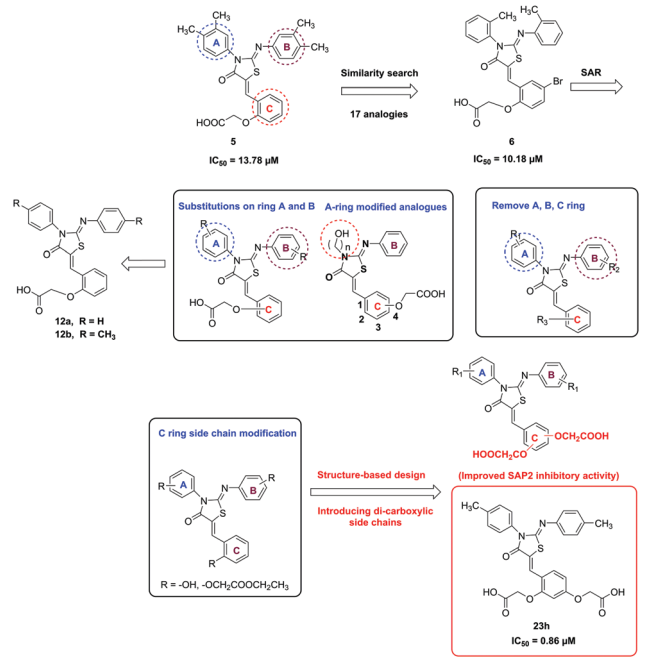

Fig. 3 Structure–activity relationship and optimization process of SAP2 inhibitor **5**.

in the SAP2 inhibitory activity was also investigated. SAP2 inhibitory assay revealed that only the 4-methyl derivative **12b** showed a slightly improved activity (IC_{50} = 9.14 μM). Removal of the A-ring or B-ring (Fig. S4 in the ESI†) and replacement of the A-ring with hydroxyl alkyl groups (compounds **18a–f**, Table S5 in the ESI†) resulted in an obvious decrease of the activity. In contrast, removal of the C-ring led to total loss of the activity (Fig. S4 in the ESI†), indicating that the C-ring played key roles in inhibitor binding. Moreover, removal or esterification of the terminal carboxyl group also resulted in inactive analogues (Fig. S4 in the ESI†), highlighting the importance of the carboxyl group. For the position of the carboxyl side chain on the C-ring phenyl group, *meta*-substituted derivatives were more potent than the corresponding *para*-position analogues because of stronger hydrogen bonding interaction with SAP2 (Fig. 4 and Fig. S5 in the ESI†). Finally, in order to improve the activity of the inhibitors, an additional carboxyl side chain was introduced in consideration of forming new hydrogen bonding interactions with the S3 pocket. Thus, a series of di-carboxyl derivatives were synthesized and assayed (Table S6 in the ESI†). Consistent with molecular docking, all the di-carboxyl derivatives showed increased SAP2 inhibitory activity. In particular, compound **23h** showed the best activity with an IC_{50} value of 0.86 μM. The additional carboxyl group of **23h** formed an additional hydrogen bond with Tyr225 (Fig. 4). Moreover, the 2,4-di-carboxyl substitution was proven to be better than the corresponding 3,4-di-carboxyl substitution.

Compound **23h** was assayed for inhibitory activity against four human aspartic proteases (*i.e.* BACE1, cathepsin B, renin and pepsin).[23] It had an IC_{50} value of larger than 100 μM towards the four enzymes, confirming its good selectivity. Human umbilical vein endothelial cells (HUVEC) were used to evaluate the growth inhibition of compound **23h** towards normal cells. To our delight, compound **23h** showed an IC_{50} value larger than 100 μM, indicating its low host toxicity. Furthermore, the *in vitro* metabolic stability of compound **23h** was evaluated using the liver microsome assay. The results indicated that compound **23h** had good metabolic stability with the $T_{1/2}$ value of 227.6 minutes.

The SAP2 gene does not affect the *in vitro* growth of *C. albicans*. Consistently, all the small-molecule SAP2 inhibitors reported herein also had no *in vitro* antifungal activity against *C. albicans* (MIC > 64 μg mL^{-1}). Thus, a series of *in vivo* studies were performed to evaluate the antifungal potency. *Candida*-mediated *Caenorhabditis elegans* assay was proven to be a facile *in vivo* model for evaluating the antifungal potency.[24] Moreover, this method can be used for concurrent evaluation for both antifungal activity and host toxicity.[24,25] As shown in Fig. 5A, the initial hit **5** was inactive at the concentration of 16 μg mL^{-1} in the nematode assay possibly because of its poor water solubility. Interestingly, compounds **12a**, **12b** and **23h** showed excellent antifungal potency. The survival rate of them (16 μg mL^{-1}) was above 60% at the end of the test, which was comparable to that of the fluconazole (FLC) group (32 μg mL^{-1}). The results confirmed that the small-molecule SAP2 inhibitors were effective in the treatment of *C. albicans* infection with low host toxicity.

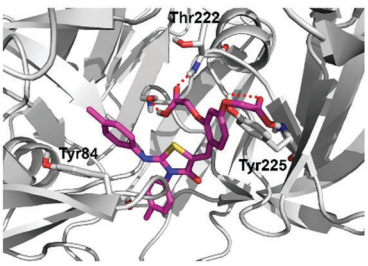

Fig. 4 Proposed binding poses of inhibitor **23h** in the active site of *C. albicans* SAP2.

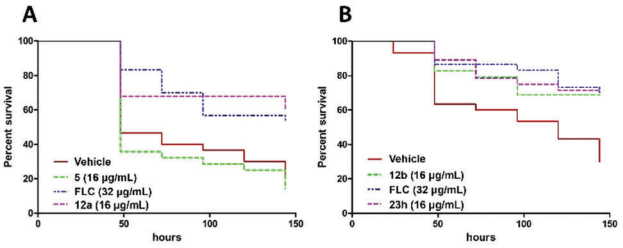

Fig. 5 Kaplan–Meier survival curves of *C. elegans* with candidiasis treated with compounds **5** (A), **12a** (A), **12b** (B), **23h** (B) and fluconazole (log-rank test: $P < 0.05$ for comparison between compounds **12a**, **12b**, **23h**, fluconazole and the blank control).

To date, the investigation for antifungal activity of virulence factor inhibitors has been rather limited. Only one report showed that peptidomimetic SAP2 inhibitors were effective in a model of vaginal candidiasis.[15] In antibacterials, virulence factor inhibitors showed therapeutic advantages in stand-alone therapy[7] or combination therapy.[5] Thus, the *in vivo* antifungal potency of compound **23h** was further explored in the models of fluconazole-sensitive and fluconazole-resistant *C. albicans* infections. ICR mice (n = 10) infected with *C. albicans* were received i.p. injections with either a drug vehicle or compound **23h** (2 mg kg^{-1} or 5 mg kg^{-1} body weight) in 24 h intervals for 7 days. The vehicle-treated mice died within 7 d, whereas animals that had received treatment with compound **23h** displayed increased time to death and 10% to 20% survival rate (vehicle *vs.* compound **23h**, $P < 0.05$; Fig. 6A and B). Although fluconazole is used as a first-line antifungal therapy in clinics, it has suffered from severe drug resistance. In the model of mice (n = 10) infected with the fluconazole-resistant clinical isolate, the fluconazole-treated mice (0.5 mg kg^{-1}) died within 14 d. In contrast, animals treated with both fluconazole (0.5 mg kg^{-1}) and **23h** (2 mg kg^{-1}) displayed a significantly increased survival rate (about 50% at the endpoint, Fig. 6C and D), indicating that the SAP2 inhibitor had synergistic effects with fluconazole. Taken together, these data demonstrated for the first time that the combination therapy of SAP2 inhibitors and fluconazole is an effective strategy to combat the drug resistance.

In summary, this report describes the discovery of the first small molecule SAP2 inhibitors as novel antifungal agents. The results presented herein have several important implications for discovery and development of SAP2-based anti-virulence agents. First, our findings provide compelling evidence that SAP2 is a tractable target for small molecule modulation.

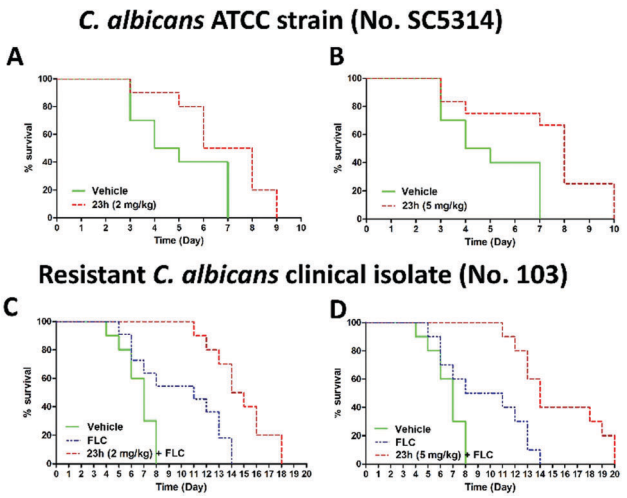

Fig. 6 *In vivo* antifungal potency of compound **23h**. (A) Kaplan–Meier survival of ICR mice infected with 0.5×10^7 CFU of *C. albicans* (ATCC strain SC5314). ICR mice (n = 10) received vehicle or compound **23h** (2 mg kg^{-1} body weight) treatment *via* i.p. injection at 24 h intervals for 7 d. Statistical significance was examined with the log-rank test (vehicle *vs.* compound **23h**, $P < 0.05$). (C) Kaplan–Meier survival of ICR mice (n = 10) infected with 0.5×10^7 CFU of *C. albicans* (fluconazole-resistant clinical isolate 103). ICR mice were treated with fluconazole (0.5 mg kg^{-1}) and a combination of fluconazole (0.5 mg kg^{-1}) and compound **23h** (2 mg kg^{-1}). Statistical significance was examined with the log-rank test (fluconazole group *vs.* combination group, $P < 0.005$).

Second, for the first time, our study demonstrates that small molecule-mediated inhibition of SAP2 can lead to therapeutic effects for the treatment of IFIs. Third, the combination therapy of the small molecule SAP2 inhibitor with fluconazole could be an effective strategy to overcome drug resistance. Taken together, these findings provide an important starting point for the design of SAP2 inhibitors as an effective antivirulence strategy to develop a new generation of antifungal agents.

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Conflicts of interest

There are no conflicts to declare.

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