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A cooperativity framework to describe and interpret small-molecule stabilization of protein–protein interactions (PPI) is presented. The stabilization of PPIs is a versatile and emerging therapeutic strategy to target specific combinations of protein partners within the protein interactome. Currently, the potency of PPI stabilizers is typically expressed by their apparent affinity or EC50. Here, we propose that the effect of a PPI stabilizer be best described involving the cooperativity factor, $\alpha$, between the stabilizer and binding partners in addition to the intrinsic affinity, $K_D^{0i}$, of the stabilizer for one of the apo-proteins. By way of illustration, we combine fluorescence polarization measurements with thermodynamic modeling to determine the $\alpha$ and $K_D^{0i}$ for the PPI stabilization of 14-3-3 and TASK3 by fusicoccin-A (FC-A) and validate our approach by studying other PPI-partners of 14-3-3 proteins. Finally, we characterize a library of different stabilizer compounds, and perform structure–activity relationship studies in which molecular changes could be attributed to either changes in cooperativity or intrinsic affinity. Such insights should aid in the development of more effective protein–protein stabilizer drugs.

Introduction

The stabilization of protein–protein interactions (PPI) using small molecules is an emerging and versatile strategy in drug development.1–3 PPI stabilizers target a specific combination of protein partners within the interactome, thereby increasing the stability of the resulting protein complex (molecular glues) (Fig. 1A).4 The direct stabilization of PPIs with small-molecules is conceptually challenging since it requires the simultaneous targeting of more than one protein within the complex.5 While several promising examples of PPI stabilizers have been reported, including Tafamidis, Rapamycin and Fusicoccin A,6 their discovery typically relied on serendipity.7–9 Therefore, rational approaches are urgently needed to assist in the development of PPI stabilizers.

The potency of a PPI stabilizer is typically expressed as a change in apparent affinity between the protein partners, either in the presence of a fixed concentration of stabilizer compound or as an EC50 of a dose-response similar to PPI inhibition.9 However, a single EC50 value does not capture the multitude of binding events in operation within the ternary complex of partner proteins and stabilizer molecule (Fig. 1B). The EC50 values depend for example on the relative concentrations of proteins and stabilizer compound. This would tend to

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Fig. 1 (A) Examples of natural compounds that stabilize protein–protein interactions. (pdb: 1FF, 1FAP, 2O98, 2O99) (B) Cooperativity scheme for PPI stabilization involving the sequential addition of PPI partner and stabilizer or vice versa.
Cooperativity, commonly expressed through a cooperativity factor $\alpha$, is a widespread phenomenon which strengthens binding within biological multicomponent systems.\textsuperscript{11,14} In GPCR research, cooperativity is used to characterize the activity of allosteric modulators\textsuperscript{15,16} and it has as well been used to describe the activity of chimeric compounds.\textsuperscript{17} To the best of our knowledge, cooperativity has yet to be established for PPI-stabilization, likely, in part, because it is more labour intensive to measure compared to direct binding measurements such as $EC_{50}$ by FP and $K_D$ by ITC for example. Also, the typical low intrinsic affinity of small molecule stabilizers for either one of the individual protein partners limits facile detection or running of simple screening assays. The correlated intrinsic affinities and the cooperativity factor in PPI-stabilization require to be determined with the support of a thermodynamic model.

PPIs involving the 14-3-3 hub proteins are widely studied. 14-3-3 are intrinsic dimers that interact with several hundred protein partners, typically through short phosphorylated motifs.\textsuperscript{18} Since many of these interactions are involved in human disease, 14-3-3 proteins have been extensively studied as drug targets,\textsuperscript{19} making 14-3-3 a useful platform to explore new types of PPI modulation, for example by natural products, stapled peptides, or supramolecular ligands and for more fundamental studies.\textsuperscript{20,21} Examples of 14-3-3 PPI partners of fundamental importance or susceptible for small molecule modulation include the cystic fibrosis transmembrane conductance regulator CFTR,\textsuperscript{22} the regulatory-associated protein of mTOR Raptor controlling cell growth,\textsuperscript{23} the bacterial ADP-ribosyltransferase toxin of Pseudomonas aeruginosa ExoS;\textsuperscript{24} and the cancer relevant targets C-Raf, ERK and p53.\textsuperscript{25-27} Here we report a theoretical framework in which the activity of PPI stabilizers for such 14-3-3 mediated PPIs can be quantified and objectively compared in a straightforward manner using two parameters, viz. the cooperativity between the stabilizer and binding partner, $\alpha$, and the intrinsic affinity of the stabilizer for the apo-protein, $K_D^{II}$. As a proof of principle, we applied this concept to revise PPI stabilization of the neuropathology relevant TWIK-related acid sensitive $K^+$-channel 3 (TASK3) and hub protein 14-3-3, by the natural product fusicoccin A (FC-A).\textsuperscript{28} Subsequently, we broaden the scope of our approach by performing binding studies of 14-3-3 proteins and other binding partners. Finally, we perform a structure–activity relationship study on a small library of FC-A analogues in terms of their intrinsic affinity and cooperativity.

**Results and discussion**

First, the affinity of TASK3 toward 14-3-3 was measured by 2D-fluorescence polarization titrations, varying the concentration of both 14-3-3 protein and stabilizer compound, FC-A (Fig. 2). In the first experiment, 14-3-3 was titrated to 10 nM FITC-labelled
Analysis of the experimental data depicted in Fig. 2 with the cooperativity model resulted in an intrinsic affinity ($K_D^{\text{in}}$) of 0.3 mM for FC-A to the apo 14-3-3σ protein and an $\alpha$-factor of 1 × 10$^{-4}$. The intrinsic affinity ($K_D^{\text{in}}$) is in the same range as previously reported using indirect measurements.$^{28}$ The resulting cooperativity factor, in contrast, is significantly larger than previously determined via classical 2D-plot analysis of the change in EC$_{50}$ value at different relative concentrations of stabilizer.$^{28}$ This observation highlights the importance of objective metrics for the characterization of PPI stabilizers.

To evaluate the broader applicability of our cooperativity model, we applied it to several other 14-3-3-binding partners, each with different affinities and FC-A sensitivities.$^{43,44}$ 14-3-3σ was titrated to each binding partner at five different concentrations of FC-A to obtain the stabilization landscape and extract the corresponding $\alpha$-factors (a selection is depicted in Fig. 4, see ESI Fig. 1† for a complete view). For some of the PPIs, the apparent affinity increased upon increasing concentrations of FC-A. For example, in the case of the CFTR-peptide the EC$_{50}$ decreased from 174 µM to 2 µM in the presence of 500 µM FC-A (Fig. 4A). Fitting of the various PPIs resulted in a range of $\alpha$-factors. The obtained intrinsic affinity ($K_D^{\text{in}}$) of FC-A for the 14-3-3 protein is, as expected, similar for all measured PPIs (around 0.3 mM). A $K_D^{\text{in}}$ of 0.3 mM was also measured in the case where FC-A functioned as a PPI inhibitor of 14-3-3/ExoS and the cooperativity of the system is in effect negative ($\alpha \approx 1$) (Fig. 4D). In the absence of, or in case of very low cooperativity ($\alpha \sim 1$) the $K_D^{\text{in}}$ could not be determined based on that individual data set alone (ESI Fig. 1†). The cooperativity of stabilizer and partner binding to 14-3-3 results in differentiated dose responses for different PPI pairs. For example, at least 100 µM FC-A is required to stabilize the 14-3-3 binding of CFTR-peptide (Fig. 4A, yellow line) while at a dose of 1 µM FC-A, the binding of the TASK3 peptide to 14-3-3 is already stabilized 20-fold (Fig. 4C, blue line). Even though the intrinsic affinity of FC-A for the apo 14-3-3 protein is of course the same and independent of the partner, stabilization of the TASK3/14-3-3 interaction occurs at a much lower stabilizer concentration due to the higher cooperativity factor in comparison to the other partners. This data makes an important point with respect to PPI stabilization, and demonstrates the value of characterizing PPI stabilizers using cooperativity. This difference in cooperativity provides a basis for selectivity in PPI stabilization. Especially for PPIs with high $\alpha$-factor values, the small molecule stabilizers can act at much lower concentrations than their intrinsic affinity for one of the binding partners alone due to the mutual enhancement of stabilizer and peptide binding. The variation in cooperativity factors between the different 14-3-3 PPIs stabilized by FC-A can be explained by studying previously published crystal structure data (See ESI Fig. 2). For example, the TASK3 protein binds such that the terminal valine residue makes a van der Waals contact with the stabilizer molecule. By contrast, the

**Fig. 3** (A) Cooperativity scheme of PPI stabilization involving sequential binding of protein (or representative peptide) partners (blue and green) and stabilizer molecule (orange). The binding partner binds to the target protein with $K_D^1$ and in the presence of a stabilizer this affinity is altered to $K_D^1/\alpha$. Similarly, the stabilizer binds with an intrinsic affinity $K_D^\text{n}$ and an enhanced affinity $K_D^\text{n}/\alpha$ when the partner is already bound to the target protein. (B) Mass action laws and mass balance equations.
With the methodology to determine the intrinsic affinity and cooperativity factor established, we used our approach to perform a structure–activity relationship study of FC-A derivatives for the stabilization of the 14-3-3/TASK3 PPI. To this end, we compiled a library of eleven fuscoecin analogues, among them FC-A and FC-J and semi-synthetic derivatives (Fig. 5 & iESI†).28,14,15 We observed that the different FC analogues each stabilized the 14-3-3/TASK3 interaction differently (Fig. 5). The α-factor and $K_D^H$ of each FC analogue was determined by fitting our numerical model to the corresponding 2D-FP titration of 14-3-3 to the labelled TASK3 (summarized in Fig. 5B). With the use of our model, a simple structure–activity relationship of PPI stabilization can thus be obtained. As previously, FC-A binds to the apo form of 14-3-3 with a $K_D^H$ of 0.3 mM. Installation of a tetrahydrofuran ring at the terpene scaffold (i.e. FC-THF), achieved through a previous semi-synthesis,28 reduced the cooperativity factor to 0.1, potentially via a steric clash with the TASK3 peptide. Small modifications to the glycone (e.g. FC-A 3 deAc and FC-A 3,19 dideAc) seem to have either a very modest or no effect on the α-factor and $K_D^H$ of FC-A, whereas complete removal of the glycone unit from FC-A substantially lowers the $K_D^H$ value towards the mM regime, with a concomitant modest reduction in α-factor. The FC-aglycone derivatives (FC-A aglycone, FC-J aglycone), and FC-THF all measured a similar moderate α-factor. This finding is interesting because in classical 2D dose-response assays, the two aglycones are detectable only at high concentrations compared to FC-A (ESI
The cooperativity paradigm thus provides an additional benefit in that it avoids false negatives; i.e. it allows the detection of weak binding stabilizers, which would otherwise be missed in conventional dose-response assays.

Fig. 3†), whereas FC-THF produces a robust response. This difference here can be explained by the different $K_a$ values. The previously published FC-NAc stabilizer has an $\alpha$-factor identical to FC-A but an increased intrinsic affinity towards 14-3-3, making FC-NAc a 30-fold more potent stabilizer, thus revealing a structural entry for further potency enhancement or hit identification.

To guide future development of PPI stabilizers, we used our model to determine the boundaries at which stabilization could be observed in terms of both cooperativity factor $\alpha$ and intrinsic affinity ($K_a$). We simulated protein titration at three different constant stabilizer concentrations (i.e. 10 μM, 100 μM, and 1 mM) for different $\alpha$’s and $K_a$ and mapped the expected enhancement in $E_{C50}$ value (Fig. 6). The corresponding position of four FC analogues are annotated as well. At a 10 μM dose of stabilizer, larger portions of the simulated space gave a less than two-fold enhancement in $E_{C50}$ value (area indicated in purple), and are therefore close to limit of detection. In this case, higher doses of stabilizer would be required to observe an effect. Indeed, an effect is observed for FC-A aglycone at 1 mM. The cooperativity paradigm thus provides an additional benefit in that it avoids false negatives; i.e. it allows the detection of weak binding stabilizers, which would otherwise be missed in conventional dose-response assays.

Fig. 6 Expected $E_{C50}$ enhancement, defined as the ratio of non-stabilized and stabilized $E_{C50}$, for protein titrations with different stabilizers concentration. The $E_{C50}$’s are determined via simulated protein titrations depending on $\alpha$ and $K_a$ at 10 μM, 100 μM and 1 mM stabilizer. The corresponding position of FC-derivatives are annotated as circles.

Conclusions
This study provides a systematic framework to interpret PPI stabilization using a cooperativity model. The model describes stabilizer efficacy in terms of its cooperativity factor ($\alpha$) as well as its intrinsic affinity towards one of the proteins ($K_a$). Both parameters are obtained in a straightforward manner through 2D-FP titrations, by varying stabilizer compound and receptor protein, in combination with a numerical model. Since absolute $K_a$’s and cooperativity factors are fundamental thermodynamic parameters, they allow for an objective comparison of different stabilizers and the establishment of structure–activity relationships between analogue stabilizer compounds. In our examples, the affinities of the stabilizers for the 14-3-3 binding partners are negligibly low, but for PPI systems where such interactions are relevant this could be added to the model.

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Notes and references

Conflicts of interest
There are no conflicts to declare.