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Mechanisms of Specific versus Nonspecific Interactions of Aggregation-Prone Inhibitors and Attenuators

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Abstract

A common source of false positives in drug discovery is ligand self-association into large colloidal assemblies that nonspecifically inhibit target proteins. However, the mechanisms of aggregation-based inhibition (ABI) and ABI-attenuation by additives, such as Triton X-100 (TX) and human serum albumin (HSA), are not fully understood. Here, we investigate the molecular basis of ABI and ABI-attenuation through the lens of NMR and coupled thermodynamic cycles. We unexpectedly discover a new class of aggregating ligands that exhibit negligible interactions with proteins but act as competitive sinks for the free inhibitor, resulting in bell-shaped dose–response curves. TX attenuates ABI by converting inhibitory, protein-binding aggregates into nonbinding coaggregates, whereas HSA minimizes nonspecific ligand interactions by functioning as a reservoir for free inhibitor and preventing self-association. Hence, both TX and HSA are useful tools to minimize false positives arising from nonspecific binding but at the cost of potentially introducing false negatives due to suppression of specific interactions.

Graphical Abstract

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Supporting Information

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Supporting Tables S1–S2 and Figures S1–S8 (PDF) Molecular Formula Strings (CSV)

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INTRODUCTION

The colloidal aggregation of organic ligands in aqueous environments poses major challenges in drug discovery and development. Aggregation-prone inhibitors are a notorious source of false positives in drug screening due to their propensity to inhibit enzymatic activity through nonspecific enzyme-aggregate adsorption.^{1–8} Such interactions modulate enzyme activity via multiple mechanisms, including unfolding, altered dynamics, and/or the physical separation of enzymes from their respective substrates.^{3,4,9–11} Aggregates may also interfere directly with the screening assay either via binding of assay reagents or interference with instrumental detection.^{12,13} Hence, it is critical to understand the molecular basis of aggregation-based inhibition (ABI) and of ABI detection and attenuation.

While nonspecific adsorption of target proteins into ligand aggregates is a recurring mechanism observed for ABI, aggregating ligands have been identified also among marketed drugs and herbal medicines that act on specific targets^{7,10} This observation has raised uncertainty about how aggregation of target-selective ligands affects the specific interactions elicited with their target receptors. In addition, it is not certain if all ligand aggregates bind proteins. Hence, it is critical to accurately detect and map the mechanisms underlying ABI as well as the specific interactions elicited by ABI-competent ligands.

Currently, detection of aggregation-prone inhibitors relies on both direct and indirect strategies. The former are based on methods such as dynamic light scattering (DLS) and transmission electron microscopy (TEM) to observe aggregate particles directly, while the latter focus on classical hallmarks of aggregation-based inhibition, such as the promiscuity toward multiple targets, increased potency with prolonged incubation time, and reduced potency in the presence of nonionic detergents, such as Triton X-100 (TX), or carrier proteins, such as human serum albumin (HSA).^{1–4,7,9,14–17}

TX and HSA are extensively utilized in high-throughput screening, as tools to detect and attenuate nonspecific interactions.^{1,2,9,15,18–21} These ABI attenuators either prevent hydrophobic compounds from aggregating or interfere with the nonspecific interactions between aggregates and proteins.^{4,22} However, it is not yet fully understood how nonionic detergents and albumin act on colloidal aggregates to reduce nonspecific interactions. In addition, it is unclear how solubilizing additives affect the free inhibitor and its specific interactions. Such effects are a major potential concern for screening, as they could compete with the specific binding of drug leads to their intended targets, resulting in false negatives. This concern is especially warranted for albumin since it is a plasma transport protein that specifically interacts with a wide variety of organic molecules, including fatty acids, small aromatic compounds, and amyloidogenic peptides.^{19,23–30} In fact, albumin is a major pharmacodynamic and pharmacokinetic determinant. Nonionic detergents could also potentially interact with free specific inhibitors by forming micelles that recruit hydrophobic inhibitors away from the aqueous solvent.

To address the open questions about the ABI mechanism as well as ABI detection and attenuation, here we focus on two prototypical hydrophobic inhibitors that target the exchange protein directly activated by cAMP (EPAC), i.e., CE3F4R and ESI-09 (Figure 1A,D). Both EPAC-selective inhibitors (ESIs) inhibit EPAC effectively and specifically at low micromolar concentrations^{31–36} and are promising pharmacological leads for treating EPAC-associated diseases, such as pancreatic cancer and cardiac hypertrophy.^{33–36} However, at higher concentrations ESIs exhibit multiple hallmarks of aggregation-based inhibition.³⁷

Herein, we investigate the aggregation equilibria of CE3F4R and ESI-09, and we examine how the resulting aggregates interact nonspecifically with EPAC and interfere with specific binding. We also investigate the molecular mechanism of action of the attenuating agents TX and HSA, as tools to minimize nonspecific aggregation-based interferences. Our investigation results in a generally applicable model for ABI and ABI-attenuation rationalized in terms of two coupled thermodynamic cycles.^{38–40} Using an experimental strategy based on the combination of biophysical techniques with progressively higher degrees of resolution (transmission electron microscopy - TEM, dynamic light scattering - DLS, surface plasmon resonance - SPR, fluorescence and ligand- and protein-NMR), we selectively probed the linked equilibria in the thermodynamic cycles for specific and nonspecific interactions, unexpectedly revealing the existence of two distinct classes of ligand aggregates with markedly different patterns of nonspecific interactions. In addition, the ABI-attenuation mechanisms of TX and HSA show how they effectively minimize false positives arising from nonspecific binding, but at the potential cost of introducing false negatives.

RESULTS

EPAC-Selective Inhibitors (ESIs) Self-Associate into Sub-Micrometer Aggregates in Aqueous Buffers.

Aggregation of CE3F4R and ESI-09 was initially examined by DLS and TEM, which are typically utilized to detect aggregates^{3,4} (Figure 1B,C,E,F). The DLS intensity profiles

identified the formation of sub-micrometer aggregates for both CE3F4R and ESI-09, with sizes in the 90–600 nm range and an average diameter close to 250 nm (Figure 1B,E). The ESI aggregates were also observed by TEM (Figure 1C,F). Interestingly, the morphology of CE3F4R aggregates differs significantly from those of ESI-09. ESI-09 aggregates exhibit a spherical micellar morphology (Figure 1F) similar to that previously reported for aggregation-prone inhibitors,^{3,4} while CE3F4R aggregates display a more amorphous morphology (Figure 1C).

The critical aggregation concentration (CAC) of the two ESIs were then measured to define the concentration ranges that result in specific 1:1 interactions vs. nonspecific aggregation-based interactions with EPAC. The concentrations of the two ESIs were increased, while monitoring changes in ¹H spectra line widths, chemical shifts, and peak intensities. Neither CE3F4R nor ESI-09 displayed any significant changes in chemical shifts, but their peak intensities, which would be expected to increase linearly with concentration in the absence of self-association, deviate from a linear correlation and reach a plateau (Figure 1G,I). The CAC was determined from the concentration at which the ESI peak intensities deviated from the initial linear correlation. In the case of both ESI-09 and CE3F4R, the CACs are ~150 μ M.

The CAC of these ESIs were independently confirmed with saturation transfer difference (STD) experiments. Since saturation transfer is only efficient for high MW complexes, the appearance of STD signals, off-resonance from the saturation frequency, is only expected for the inhibitor aggregates.^{41,42} At concentrations below the CAC, as determined by the intensity analyses (Figure 1G,I), no significant STD signals were observed for CE3F4R or ESI-09, other than those arising through chemical exchange (Figure 1H,J; blue spectrum). When the ESI concentration was increased to the CAC or higher, STD signals arise for other resonances (Figure 1H,J; green and purple spectra). The combined analyses of the DLS, TEM, and NMR data consistently indicate that the ESIs, CE3F4R and ESI-09, both form large colloidal aggregates with diameters of hundreds of nanometers and CACs around 150 μ M.

Different ESI Aggregates Interfere with EPAC-Specific Interactions Through Diverse Mechanisms.

To investigate the types of interactions between EPAC and the ESIs that could occur both below and above their CACs, we monitored the ESI titrations through chemical shift changes and intensity losses in the NH-HSQC spectra of the uniformly ¹⁵N-labeled cAMP-binding domain (residues 149–318) of EPAC1 (EPAC1_{CBD}). CE3F4R, a unique uncompetitive inhibitor,^{31,33,34} binds to the cAMP-bound state of EPAC1_{CBD} resulting initially in significant chemical shift changes (Figure 2A,B). The chemical shift changes are both residue dependent and multidirectional within the (¹H,¹⁵N) plane, as typically expected for specific interactions (Figure 2A,B).^{43–50} In contrast, nonspecific interactions or solvent effects often result in unidirectional chemical shift changes with reduced residue-dependence, such as those observed for DMSO (Figure S1A,B).

Interestingly, when the concentration of CE3F4R is increased beyond 300 μ M, which is a value significantly higher than its CAC, the chemical shifts revert toward the CE3F4R-free

state (Figure 2A,B). These chemical shift changes likely reflect a dissociation event, since all residues shift toward the CE3F4R-free state by a similar degree and in a linear manner with respect to the initial binding event (Figure 2A,B). The dissociation of CE3F4R is presumably due to CE3F4R aggregates binding and sequestering free CE3F4R in a competitive manner to EPAC1_{CBD}. The dissociation event occurs at CE3F4R concentrations that are significantly higher than its CAC (~150 μ M), because EPAC1_{CBD} solubilizes a fraction of CE3F4R through specific binding.³¹ Therefore, higher concentrations than the CAC of CE3F4R are required to promote aggregation.

The analysis of the CE3F4R NH-HSQC titration data also suggests that EPAC1_{CBD} does not interact with CE3F4R aggregates. Since the aggregates are hundreds of nanometer in size, any interaction with EPAC1_{CBD}, even one that is transient or lowly populated, would cause substantial line broadening and intensity losses of EPAC1_{CBD} peaks.^{51,52} However, the intensity loss of EPAC1_{CBD} in the presence of CE3F4R was no more than that caused by DMSO used as a carrier solvent (Figure S1C,D and Figure 2G). DLS volume scattering profiles provide additional evidence that confirms EPAC1_{CBD} does not interact with CE3F4R aggregates. Apo EPAC1_{CBD} appears around 4.2 nm (Figure 2H, black), which matches the size expected based on X-ray crystal structures.^{53,54} Addition of CE3F4R, at concentrations either below or above its CAC, does not result in any major change in the volume profile of EPAC1_{CBD}, confirming that the CE3F4R aggregates do not interact with EPAC1_{CBD} (Figure 2H).

In contrast to CE3F4R, ESI-09 displays attributes commonly expected for aggregation-prone inhibitors that nonspecifically adsorb target proteins. At concentrations below its CAC, ESI-09 interacts specifically with EPAC1_{CBD}, as seen by the significant residue-dependent and multidirectional chemical shift changes in NH-HSQC spectra (Figure 2C,D). However, ESI-09 also causes substantial signal losses for EPAC1_{CBD} peaks (Figure 2C,G and Figure S1E,F).

Such signal reduction is present even below ESI-09's CAC, suggesting that some loss is due to intermediate chemical exchange broadening. The exchange broadening arises from specific binding, as suggested by the residue-dependence of the intensity losses, which are especially predominant in the base binding region (BBR) and phosphate binding cassette (PBC) (Figure S1F), two motifs where ESI-09 is expected to bind specifically, based on its competitive nature with cAMP.

Chemical shift perturbations were also used to measure the affinity of ESI-09 for EPAC1_{CBD}, yielding a K_d of about 20 μ M (Figure 2E). The effective K_d of ESI-09 was additionally measured through a competitive experiment with a fluorescently labeled cAMP molecule (8-NBD-cAMP).^{35,55,56} This fluorescence competition method was validated through the measurement of the K_d for cAMP (4.5 μ M; Figure 2F), which is similar to previously reported values.⁵⁷ The competition experiment with ESI-09 creates a biphasic curve with an inflection point around the CAC of ESI-09 (Figure 2F), suggesting that the sudden drop in fluorescence is due to aggregate interference. Fitting of the curve using points below the CAC yields an effective K_d of 20 μ M (Figure 2F), in full agreement with the ESI-09 titration monitored by NMR chemical shifts (Figure 2E).

When the ESI-09 concentration is increased above its CAC, the EPAC1_{CBD} signal losses becomes more pronounced and less residue dependent, pointing to significant nonspecific interactions of EPAC1_{CBD} with the ESI-09 aggregates (Figure 2G). The interactions of EPAC1_{CBD} with ESI-09 aggregates are also observed by DLS (Figure 2I). When EPAC1_{CBD} is in the presence of ESI-09 at concentrations above its CAC, its size profile increases by up to 5 nm (Figure 2I), which is too large to arise from conformational changes. However, the size is also much smaller than the size of ESI-09 aggregates (~250 nm), indicating that the observed peak is likely a population weighted average of free and aggregate bound EPAC1_{CBD}.

Overall, our analyses of EPAC1_{CBD}:ESI interactions reveal the existence of diverse mechanisms of aggregation-based interferences. Most aggregation-prone inhibitors participate in two competing equilibria, one in which the inhibitor interacts specifically with its intended target receptor, and one in which the inhibitor associates into large colloidal aggregates (Figure 2J). When the concentration of free inhibitor is below the CAC, only specific interactions are observed, while above the CAC the aggregation equilibrium is present. Once aggregates begin to emerge, two different aggregation-based interferences can develop. The more classic interference, which has previously been described, is the nonspecific interaction of target proteins with aggregates (Figure 2J), resulting in altered protein structure and/or function.^{3,4,9,11} This mechanism is exemplified here by ESI-09, as at high concentrations it causes absorption of EPAC1_{CBD} and possibly its unfolding.³⁷ Alternatively, ligand aggregates do not interact with target proteins, as illustrated by the case of CE3F4R. However, this class of inert aggregates can still generate interferences by competing with the target protein for the free ligand. If the affinity of the free inhibitor for the aggregates is greater than for the intended target receptor, it will cause an apparent dissociation from the target receptor and consequent losses of specific interactions. Regardless of whether the inhibitor aggregates are promiscuous (i.e., nonspecific binders of the target protein), as in the case of ESI-09, or inert competitors for free inhibitor, as in the case of CE3F4R, aggregation imposes major challenges for drug screening and characterization. Hence, in the following sections, we investigate the use of solubilizing agents, such as Triton X-100 (TX) and human serum albumin (HSA), as tools to minimize the nonspecific effects of inhibitor aggregation.

Investigation of Triton X-100 (TX) as an Agent to Attenuate ESI Aggregation.

TX was examined as a tool for minimizing nonspecific interferences caused by ESI aggregation. However, a concern with using TX to solubilize inhibitor aggregates is that it is a micelle forming compound that may interact with proteins. For instance, TX is commonly used for extraction of membrane proteins and has also been observed to interact with soluble proteins such as albumin.^{58–60} In addition, TX micelles could provide a hydrophobic environment that sequesters free ESI ligands, thereby reducing their availability for EPAC1_{CBD}. The critical micellar concentration (CMC) of TX is around 0.22–0.24 mM (0.013–0.014%).⁶¹ In theory, TX concentrations below the CMC should be used. However, the concentration range of TX that is typically utilized for the solubilization of aggregating inhibitors is between 0.01% and 0.1%.^{1,2,4,9,22} While TX concentrations above 0.01% are less common than those at or below 0.01%, this means that TX micelles may be present

under conditions used for eliminating aggregate interactions (Figure S2). Therefore, we investigated how TX micelles form and whether they interact with the EPAC1_{CBD} and the ESIs.

We initially examined direct interactions between EPAC1_{CBD} and TX by probing NH-HSQC chemical shift and intensity changes similar to the ESI interactions in the previous section. The addition of TX, up to concentration of 0.1% (well above CMC), causes no significant chemical shift changes or intensity losses for either apo or cAMP-bound EPAC1_{CBD} (Figure S3). These findings show that, at these concentrations, TX does not significantly perturb the structure of EPAC1_{CBD}, which suggests that TX micelles are inert with respect to EPAC1_{CBD} binding, similar to CE3F4R aggregates.

To characterize the mechanism of aggregate disruption by TX, we developed an approach to monitor micelle formation of TX that could be used concurrently with approaches to probe ESI aggregation. ¹H spectra line widths, chemical shifts, and peak intensities were monitored at different TX concentrations (Figure 3A,B), similar to the ESIs in the previous sections (Figure 1). At concentrations of 0.01% or less, TX produces sharp peaks with little chemical shift changes (Figure 3A,B). Beyond 0.01%, TX peaks broaden significantly and shift in position (Figure 3A,B). These observations are indicative of TX micelle formation. Micelle formation is also supported by STD and DLS experiments, which confirm that TX micelles develop at concentrations above 0.01% (Figure S2). TX micelles are also significantly smaller in size than the ESI aggregates, with an average diameter of about 10 nm (Figure S2C,D). The CMC value of 0.013% (~225 μ M, Figures 3A,B and S2) is also consistent with values that were previously reported for TX.⁶¹ In addition, all these approaches can be used to investigate aggregate formation of TX in the presence of the ESIs, since there is sufficient resolution to distinguish between the TX and ESI signals.

Interactions between TX and the ESIs were first examined at concentrations below each of their respective CAC/CMCs, i.e., under conditions in which the isolated TX or ESIs do not form micellar aggregates. The combination of 0.010% TX with 100 μ M CE3F4R causes significant chemical shift changes for both compounds and line broadening of TX peaks similar to that observed for TX at micellar concentrations (Figure 3C, green spectra). CE3F4R also exhibits line broadening for most peaks, but not to the same degree as TX. In addition, a new slow exchanging equilibrium is observed, as indicated by the appearance of new peaks, which were assigned using ROESY NMR to a complex formed by TX and CE3F4R (Figure 3C; Figure S4E). The residual CE3F4R signals in the 1D spectra reflect an abundant free form in dynamic equilibrium with a minimally populated TX bound form. These results suggest that CE3F4R interacts with TX and forms coaggregates. Some protons of CE3F4R were influenced by this interaction more than others. For instance, the formyl resonance (C1) of CE3F4R did not significantly change from its free form in either chemical shift or line width (Figure 3C), whereas the C3, C4, and C5 resonances all broadened and shifted to lower ppm values (Figure S4A). This could indicate that the formyl moiety of CE3F4R binds at the surface of the TX:CE3F4R co-micelles, while the opposite side is embedded within the hydrophobic core. The interaction also appears to be selective for the cis isomer of CE3F4R. As described previously,^{31,62} CE3F4R samples a pre-existing slow exchanging equilibrium caused by cis/trans isomerization of the formyl moiety (denoted

with C and T subscripts, respectively). The C6 resonance of the trans isomer only shifts by about 0.02 ppm upon addition of TX, while the C6 resonance of the cis isomer shifts by up to 0.34 ppm. This is an interesting finding considering that EPAC1_{CBD} preferentially interacts with the trans isomer.³¹

Combining 0.01% TX with 100 μ M ESI-09 also causes chemical shift changes, line broadening, and the appearance of new peaks in proton NMR spectra (Figure 3D, green spectra). The new peaks are limited to ESI-09, arising from the splitting of the E2 resonance (which represents three protons in the chlorophenyl ring) into three discrete signals (Figure S4B). Overall, the findings suggest that TX interacts with the ESIs creating heterogeneous micelles at concentrations below the CAC/CMC of the respective compounds in isolation. Dilution of the ESI and TX concentrations restores the peaks to their original chemical shifts and line widths, showing that the interaction is reversible and not present at low concentrations where micelles/aggregates are absent (Figure 3C,D).

The formation of heterogeneous aggregates composed of TX and the aggregation-prone ESIs provides a novel mechanism to explain inhibitor sensitivity to detergents. Therefore, we further investigated if these types of TX-inhibitor interactions were unique to these ESIs or more ubiquitous among aggregating inhibitors. Our initial screen examined TX interactions with four representative commercially available drugs, which were previously classified as strong aggregators: benzyl benzoate, clotrimazole, miconazole, and nicardipine (Figure S5A).⁷ All four compounds interact with TX, as indicated by 1D chemical shift changes, line broadening, and/or intensity losses (Figure S5B–E). Interestingly, the interaction of miconazole with TX causes extreme line broadening of the TX resonances as well (Figure S5D). We also investigated whether TX interacts with nonaggregating compounds that are hydrophobic in nature. These include the drugs diflunisal and lamotrigine, as well as the fluorescent dye ANS, which have all been described as nonaggregators previously (Figure S6A).⁷ Both diflunisal and ANS experience chemical shift changes and line broadening in the presence of TX (Figure S6B,D). Lamotrigine, which is the most soluble of the three, does not undergo any significant chemical shift changes, but does experience minor line broadening for selected peaks (Figure S6C, inset). We also examined two other hydrophobic drugs, ibuprofen and naproxen, and the polar allosteric EPAC effector, cAMP, but found no evidence that they interact with TX (Figure S6E–G). Overall, these results indicate that TX interacts with a variety of hydrophobic compounds, including both aggregators and nonaggregators. It is notable that these interactions involve also known drugs, suggesting that specific, nonaggregating inhibitors may also be sensitive to detergents in biochemical drug screens.

To further investigate the nature of interactions between TX and the ESIs, we utilized STD and transfer NOESY experiments. The STD experiments were performed similarly to the ones described for Figure 1H,J. In these experiments, either TX or the ESI was selectively irradiated, and saturation transfer was observed both intra- and intermolecularly. As expected, the saturation of either TX or the ESI resulted in both intra- and intermolecular STDs (Figure S4C,D). This finding confirms that the ESIs coaggregate with TX. Transfer NOESY experiments were also acquired for both CE3F4R and ESI-09 in the presence of TX (Figures 3E and S4E,F). The combination of TX and CE3F4R generates both intermolecular

NOEs and intramolecular NOEs with the same sign as the diagonal (Figure S4E). As noted earlier, the combination of TX and CE3F4R creates a new slow exchanging equilibrium, marked by the appearance of new peaks (Figure S4E). The new peaks include intermolecular NOEs between CE3F4R and TX, which likely reflect the compounds in their coaggregated forms, denoted with b (bound) subscripts (Figure S4E). The observed intermolecular NOEs are compiled in Table S1 and are represented graphically in Figure 3E. The formyl group (C1) exhibits the fewest, and most often, the weakest intermolecular NOEs with TX, with its strongest NOEs being with TX's T3 group. The C6 proton, which is on the same face of CE3F4R, also exhibits its strongest NOEs with the T3 group and other strong NOEs with the nearby T4 and T5 resonances in the TX's aromatic ring. In contrast, the C3, C4, and C5 groups display their strongest NOEs with the TX's T7 resonance. These findings suggest that CE3F4R is imbedded into TX micelles near the TX's aromatic ring with its formyl (C1) and aromatic (C6) protons facing toward the surface, while the C3, C5, and C6 protons face toward the center of the micellar inner core (Figure 3E). Another notable feature of TX:CE3F4R coaggregates is that over the duration of the experiment (~14 h), the slow exchanging equilibrium between free and bound states transitioned to fast exchange, as seen in the 1D spectra (Figure S4E, red 1D spectrum). This effect points to the existence of an initial kinetic barrier to the formation of the heterogeneous TX:CE3F4R coaggregates, which then decreases over time.

A similar analysis was performed for ESI-09 and TX. The combination of these molecules does not result in an initial slow exchanging equilibrium, unlike CE3F4R, but it does generate transfer intermolecular NOEs and positively phased intramolecular NOEs (Figure S4F). The t-butyl group (E4) of ESI-09 displays the most intermolecular NOEs with TX (Figure 3E, Figure S4F; Table S2). Its strongest intermolecular NOE is with the T3 group of TX, and its other intermolecular NOEs decrease in strength as the distance from the T3 group increases. The other ESI-09 groups (E1–E3) also result in strong NOEs to either the T3 or T5 groups, indicating that ESI-09 likely interacts with TX's aromatic ring.

To provide an external validation of the NMR results about the TX:ESI interactions, DLS experiments were performed to detect the TX:ESI coaggregates and to probe their morphological changes relative to the micelles/aggregates of the ESIs and TX in isolation. The ESIs were mixed together with TX at roughly a 1:1 ratio using concentrations that were below their respective CACs/CMCs, as indicated by their 1D line width and intensity analyses (Figure 3A,B; Figure S2). The combination of CE3F4R and TX at a 1:1 ratio creates an unstable heterogeneous mixture of different sized aggregates (Figure 3F, blue). The reading was recorded as five measurements, but each resulted in a different intensity profile (Figure S7A). The average profile contains attributes of both TX and CE3F4R micelles/aggregates, with one peak around 10 nm in size and another around 100 nm in size (Figure 3F, blue). When the ratio of TX to CE3F4R was increased to 1:2, the scattering profile became more homogeneous with a single peak that had an average size of around 70 nm (Figure 3F, green). In contrast, the mixture of ESI-09 with TX at a 1:1 ratio creates a homogeneous intensity scattering with a size similar to TX micelles (Figure 3G, blue). When the ESI-09 concentration is doubled, the peak around the TX position decreases slightly in size, and a new peak appears around the size of ESI-09 aggregates (Figure 3G, green).

Overall, the DLS analysis confirms that mixing TX with ESIs, even at concentrations below the respective CAC/CMC values, causes formation of aggregate species in solution. The TX:CE3F4R coaggregates appear larger in size than TX micelles (~10 nm) but considerably smaller than the isolated CE3F4R aggregates (~250 nm). Whether this is because the true size of these particles is within this range or because the peak represents a population weighted average of a fast exchanging equilibrium between TX-sized and CE3F4R-size aggregates remains uncertain. However, the DLS data confirm that the size distributions of the TX:CE3F4R coaggregates are different than those of the isolated compounds, while the coaggregation of TX and ESI-09 produces particles with a morphology more similar to the isolated TX.

Determination of TX's Mechanism of Action.

Our cumulative NMR and DLS analyses suggest a viable hypothetical mechanism by which TX disrupts aggregation-based interferences: TX causes the loss of inhibitory activity by forming inert coaggregates with aggregating inhibitors that prevent them from interacting with their target receptors, either specifically or nonspecifically. To further test this hypothesis, we examined how TX influenced interactions, both specific and nonspecific, of the ESIs with EPAC1_{CBD}. Since CE3F4R aggregates do not interact with EPAC1_{CBD}, it was used as a probe to investigate whether TX could competitively displace CE3F4R specifically bound to EPAC1_{CBD}. The addition of TX to a sample of EPAC1_{CBD} prebound with CE3F4R causes HSQC peaks to shift toward the CE3F4R-free state (Figure 4A,B). This effect supports the hypothesis that TX acts as a competitive sink dissociating CE3F4R from EPAC1_{CBD}. Similarly to CE3F4R, the addition of TX to EPAC1_{CBD} prebound with ESI-09 causes dissociation of ESI-09 (Figure 4C). However, it also reverses the signal losses caused by ESI-09 binding. Since the concentration of ESI-09 in this sample was below its CAC, these restored intensity losses likely reflect the removal of the chemical exchange broadening caused by its specific binding.

To determine if TX could act in a protective manner to block EPAC1_{CBD} interactions with ESI-09 aggregates, NH-HSQC intensity losses and chemical shift changes were monitored, in the presence of 0.025% TX, in response to an ESI-09 titration reaching concentrations above its CAC. The addition of ESI-09, even at concentrations up to 200–300 μ M, results in relatively insignificant chemical shift changes (Figure 4D). However, EPAC1_{CBD} peak intensities are substantially decreased similarly to the signal losses observed in the absence of TX (Figure 4D,E). These data suggest that, under our experimental conditions, TX not only significantly reduces specific 1:1 interactions of ESI-09 with EPAC1_{CBD}, but it also offers no additional protection of EPAC1_{CBD} from nonspecific interactions with ESI-09 aggregates. A possible explanation of this observation is provided by the DLS data in Figure 3G, revealing that the mixture of 2:1 ESI-09:TX produces two types of aggregates similar in size to either isolated TX micelles or ESI-09 aggregates (Figure 3G). The larger particles were assumed to be homogeneous ESI-09 aggregates that appeared following saturation of the TX:ESI-09 coaggregates, which would explain why TX offers no significant protection from aggregation-induced intensity losses in HSQC spectra during the ESI-09 titration. However, the aggregation-induced intensity losses were substantial even when the concentration of ESI-09 was less than TX. Therefore, we investigated if the aggregates that

cause line-broadening in the HSQC experiments were only homogeneous complexes of ESI-09 or also heterogeneous coaggregates of TX and ESI-09 with chemical properties similar to ESI-09 aggregates.

The nature of the TX aggregates was investigated by measuring changes in ^1H chemical shifts, line widths, and peak intensities (Figure 4F). As the concentration of ESI-09 was increased, the peaks of TX shifted and broadened, eventually beyond detection (Figure 4F). This suggests that TX is being incorporated into ESI-09-like coaggregates and that the relative abundance of the two components in the coaggregates may dictate their properties. The extreme broadening of TX signals was also noted in the 1D analysis of miconazole (Figure S5D), which was acquired with an excess of TX ($2.25\times[\text{miconazole}]$). This may indicate that the properties of TX coaggregates also potentially depend on the characteristics of the ligand.

We also investigated whether the ESI-09-mediated EPAC1_{CBD} signal loss could be reversed by adding more TX to shift the properties of the aggregates back toward TX-like micelles. The addition of TX, up to a concentration of 0.1%, recovers some peaks in the HSQC spectra, but the effect is relatively minor in comparison to aggregate induced signal losses (Figure 4G). Despite these limitations of TX (e.g., prevalence of large colloidal aggregates and interactions with nonaggregating compounds), TX remains a useful tool for detecting and minimizing aggregation-based interferences by acting as an inert reservoir for hydrophobic inhibitors. However, TX requires careful optimization to balance losses in specific vs nonspecific interactions. Therefore, in the next section HSA is investigated as an alternative strategy to attenuate ABI.

Investigation of Human Serum Albumin (HSA) as a Tool to Attenuate ESI Aggregation.

As an initial step to assess whether HSA behaves as an ESI reservoir that eliminates inhibitor aggregation, interactions between HSA and the ESIs were investigated by 1D ^1H NMR (Figure 5A,B). HSA causes extreme line broadening of ESI signals even when its concentration is 20–40 $\times$ lower than the ESIs (Figure 5A,B). This finding confirms that the ESIs interact with HSA. To determine how the HSA:ESI binding equilibrium is potentially coupled to the ESI self-association and EPAC:ESI binding equilibria, we explored the binding stoichiometry and affinities of the HSA:ESI interactions. The location of ESI binding sites within HSA was determined through ^{13}C -oleic acid (OA) competition experiments. OA binds to HSA's fatty acid (FA) binding sites and its two drug binding sites, Sudlow sites 1 and 2 (denoted as SS1 and SS2, respectively), resulting in unique CH-HSQC cross-peaks.^{25,63,64} The binding to any of these sites of a ligand that displaces the OA leads to signal losses of selected CH-HSQC cross-peaks (Figure 5C,D). Both CE3F4R and ESI-09 displace OA at multiple sites (Figure 5C,D), indicating the presence of multiple ESI binding sites within HSA. The most significant peak intensity losses for the two ESIs are at the two Sudlow's drug binding sites of HSA (peaks D and F in Figure 5C,D).

The affinities of the ESIs for the two Sudlow's sites were measured using fluorescence competition experiments with dansylated arginine (DanR) and phenylalanine (DanF). DanR specifically targets Sudlow's Site I with a K_d of about 25 μM (Figure S8A), while DanF targets both Sudlow sites (Figure S8B).³⁰ At concentrations below its CAC, CE3F4R is

ineffective at displacing either of the dansylated amino acids from HSA (Figure S8C), pointing to a weak affinity for HSA. In contrast, ESI-09 is able to displace both dansylated amino acids with apparent affinities for Sudlow sites I and II of 230 and 99 nM, respectively (Figure 5E,F). Interestingly, these apparent K_d values, which represent only upper limits for these sites, are two orders of magnitude lower than the K_d for EPAC1_{CBD}. Interactions of the ESIs with other sites on HSA were examined by ANS fluorescence competition experiments. Under our experimental conditions ANS binds up to four sites on HSA with low micromolar affinity (Figure 5G), consistent with previous reports suggesting that ANS binds the two Sudlow's sites as well as up to three additional sites.^{30,65–67} While CE3F4R is unable to displace ANS at concentrations below its CAC (Figure S8D), ESI-09 can fully displace even large excesses of ANS at low micromolar concentrations (Figure 5H). Taken together, the HSQC OA and the DanR, DanF, and ANS fluorescence competition data consistently indicate that ESI-09 binds multiple sites on HSA with high affinity.

On the basis of these results, we examined if the HSA:ESI interactions disrupt ESI aggregation. Due to spectral overlap and the extreme line broadening caused by binding, the NMR approaches that were used previously to monitor aggregation could not be employed. Therefore, we reverted to surface plasmon resonance (SPR) as a means for probing inhibitor aggregation^{68,69} through the nonspecific binding of ESI aggregates to a nonfunctionalized gold nanoparticle sensor. To determine if ESI aggregates could be detected in this manner, the ESIs were injected at concentrations both below and above their CAC. There was no significant change in SPR signal detected in the presence of CE3F4R, relative to the DMSO control, at either of its concentrations (Figure 6A), but we observed a significant difference for ESI-09 when injected at a concentration that is above its CAC (Figure 6B). This observation suggests that the SPR approach is effective in tracking ESI-09 aggregation, but not CE3F4R, in agreement with the different nature (i.e., inert vs. invasive) of CE3F4R vs. ESI-09 aggregates.

Next, we utilized SPR to investigate whether the binding of ESI-09 to HSA prevents aggregation. The combination of 250 μ M ESI-09 with 25 μ M HSA results in no significant SPR signal increase relative to the corresponding HSA and DMSO control (Figure 6C). To ensure that this result reflects a genuine loss of ESI-09 aggregation and is not due to saturation of the gold nanoparticles by HSA, the experiment was repeated with 500 μ M ESI-09 (Figure 6D). Under these conditions, the SPR signal significantly increases beyond the HSA and DMSO reference, confirming that the sensor was not saturated and that the ESI-09 aggregates reappear only after HSA is fully saturated and the free ESI-09 concentration exceeds the CAC. If HSA includes up to nine binding sites for ESI-09,^{63,64} it can potentially bind up to 225 μ M of ESI-09 in these samples, explaining why aggregation is absent with 250 μ M ESI-09 and 25 μ M HSA, but not with 500 μ M ESI-09 and 25 μ M HSA. To independently confirm these conclusions, ESI aggregation in the presence of HSA was also monitored by DLS. For both CE3F4R and ESI-09, there are no detectable aggregates in the presence of HSA nor is there any significant change in the position of the HSA scattering profile (Figure S7B,C).

Once we established that HSA prevents ESI aggregation, we investigated HSA as a protective agent alternative to TX to study specific EPAC1_{CBD}:ESI interactions. As

expected, given its interactions with the ESIs, HSA acts in a competitive manner to EPAC1_{CBD} and increases the concentration of CE3F4R required to achieve similar levels of EPAC1_{CBD} binding relative to conditions devoid of HSA (Figure 7A). Similarly, the titration of EPAC1_{CBD} with CE3F4R shows that HSA increases the apparent K_d for EPAC1_{CBD} binding by 5–10 fold (Figure 7B). However, an advantage of HSA is that it permits the use of concentrations of CE3F4R that would otherwise aggregate and competitively sequester monomeric CE3F4R away from EPAC1_{CBD} (Figure 7A,B). In the absence of HSA, CE3F4R dissociation from EPAC1_{CBD} occurs at a concentration above about 250–350 μ M, but in the presence of HSA, the dissociation starts at concentrations $\sim$ 750 μ M (Figure 7B). While this result indicates that HSA may serve as a promising protective agent that attenuates aggregation-based interferences, it also shows that HSA increases the concentration of CE3F4R required to achieve similar fractional saturations of EPAC1_{CBD} by CE3F4R compared to samples without HSA. This is likely because the affinity of CE3F4R for HSA is either tighter or similar to the affinity for EPAC1_{CBD}.

The protective effects of HSA were also investigated with respect to the EPAC1_{CBD}:ESI-09 interactions. Similar to CE3F4R, the HSA's interactions with ESI-09 causes competition with EPAC1_{CBD}, but they also reduce signal losses caused by the binding of ESI-09 aggregates to EPAC1_{CBD} (Figure 7C). This conclusion is further confirmed by the comparative analysis of ESI-09 titrations performed in the presence and absence of HSA (Figure 7D). For most ESI-09 concentrations, the EPAC1_{CBD} HSQC intensities are 30–40% higher in the presence of HSA, showing that HSA elicits a significant protective effect due to the prevention of ESI-09 aggregation by HSA. However, specific binding is also drastically reduced due to competitive and high affinity binding of ESI-09 to HSA. For instance, the chemical shift changes at 100 μ M ESI-09 in the presence of HSA are less than $\sim$ 20% of those in the absence of HSA (Figure 7E). Even at an ESI-09 concentration that yields similar intensity losses to the sample in the absence of HSA, the average ESI-09-induced chemical shift changes are still $\sim$ 30% less than those observed in the absence of HSA (Figure 7E).

DISCUSSION

Thermodynamic Cycle of Aggregation-Based Inhibition (ABI).

Through an experimental strategy based on the combination of ligand- and protein-NMR, fluorescence, SPR, DLS, and EM, we have shown that one of the simplest models for the mechanism of ABI is a four-state thermodynamic cycle (Figure 8). The cycle arises from the coupling of aggregation/nonspecific adsorption equilibria with the equilibrium for the specific binding of the monomeric inhibitor (ESI) to its respective target protein (EPAC; Figure 8). Two key determinants of these equilibria are the CAC and the inhibitor's dissociation constant ($K_{d,EPAC}$). The CAC of a given inhibitor is accurately measured by combining NMR, DLS, and EM (Figure 1), while the $K_{d,EPAC}$ for the specific inhibitor–target complex can still be determined in the presence of nonspecific interactions by relying on protein chemical shift changes (Figure 2A–E) and fluorescence competition (Figure 2F). If the CAC of the inhibitor is less than $10 \times K_{d,EPAC}$, as it is often the case for drug leads with moderate to weak affinities for their targets, saturation of the specific binding sites requires

concentrations of free inhibitor above the CAC. Under these conditions, aggregation effects are not negligible, and the inhibitory function of the ESI needs to be rationalized in the context of the full four-state thermodynamic cycle, which may include the nonspecific adsorption of EPAC1_{CBD} into the ESI aggregates (Figure 8). The latter is best probed through losses in NMR signal intensities of EPAC1_{CBD} (Figure 2G) and changes in DLS profiles (Figure 2H,I).

Two Distinct Types of Aggregation-Based Inhibitors.

The combined analysis of NMR intensity losses (Figure 2G) and DLS (Figure 2H,I) reveals two clearly distinct types of aggregation-based inhibitors with diverse morphologies (Figure 1C,F) and ABI mechanisms (Figure 8, bottom grid). Type A inhibitors, such as CE3F4R, form inert aggregates with negligible protein adsorption, while type B inhibitors, such as ESI-09, self-associate into invasive aggregates that adsorb the target protein and result in nonspecific inhibition. However, both types of aggregates act as sinks of monomeric ESI as the concentration of free inhibitor increases beyond the CAC, leading to a depletion of specific inhibitory complexes (Figure 8, bottom grid). Such a decrease in the population of the EPAC1_{CBD}:ESI specific complex explains the unexpected and unusual reversal in the EPAC1_{CBD} chemical shift changes observed for the CE3F4R titration (Figure 2A) and implies that for both types of inhibitors, aggregation competes with specific interactions. This finding may also explain the nature of bell-shaped dose–response curves that are often observed for drugs and other biochemical reagents.^{70,71} This in turn means that, while aggregation-based inhibitors of type B may produce false positives in drug screens, those of type A may lead to false negatives. Both types of aggregation-based inhibitors pose major challenges for structural characterizations of specific binding sites and mechanisms of action. In order to minimize such interferences arising from ABI, it is critical to understand the molecular mechanisms of commonly utilized aggregation attenuators, such as nonionic surfactants (e.g., TX) and carrier proteins (e.g., HSA).

Mechanisms of Aggregation-Based Attenuation.

A simple but effective means to model the interactions between TX and aggregation-based inhibitors is another thermodynamic cycle in which the inhibitor self- and coaggregation equilibria are coupled (Figure 8). The ESI:TX coaggregation equilibrium was probed by combining DLS with NMR (i.e., ¹H chemical shift and intensity changes, intermolecular NOEs and STD), as shown in Figure 3 and Figure S4, revealing that TX forms coaggregates by interacting directly with hydrophobic compounds, including both aggregators and nonaggregators (Figures S5 and S6). Co-aggregation of these compounds with TX reduces the CAC of the complex in comparison to the CACs of the respective isolated components. Since TX micelles are inert with respect to protein binding, the coaggregates between TX and the aggregating inhibitors have the potential to become inert, even for type B ligands. However, this property is partially dependent on the composition of the TX/inhibitor coaggregates, as when type B aggregating inhibitors become the predominant species, the coaggregates also become invasive, as shown in Figure 4.

Unlike TX, HSA prevents aggregate formation rather than remodeling invasive type B aggregates into inert type A coaggregates (Figure 8). HSA acts as an inherently inert

reservoir that preferentially binds the monomeric inhibitor, hence preventing ESI aggregation (Figure 5; Figure S8). Through competitive displacement of isotopically labeled and fluorescent ligands monitored by NMR and fluorescence, respectively, the affinities and stoichiometries of the HSA-inhibitory complex were determined, revealing the presence of multiple high affinity ESI-09 binding sites within HSA (Figure 5; Figure S8). A common feature shared by TX and HSA is that both agents compete with the specific binding of the inhibitor to its target receptor. Such competitive displacement of the inhibitor from its target leads to a depletion of specific interactions (Figures 4 and 7).

Hence, while TX and HSA are excellent tools to minimize false positives arising from ABI of type B in ligand screens, they may also introduce false negatives. For instance, there are several ligands among commercially available drugs that interact with TX. If they were evaluated on the basis of their interactions with TX, they may have not been recognized as drugs leads. This false positive vs false negative balance should be considered when interpreting results from ligand screening campaigns implemented in the presence of ABI attenuating agents. The mechanisms proposed here for ABI and its attenuation (Figure 8), as well as the related experimental strategy, will assist in such interpretation and in finding conditions under which TX and HSA minimize nonspecific aggregation-based interferences, while maintaining specific receptor interactions. While it is clear that the specific CAC and affinity values vary from system to system, the proposed mechanism (Figure 8) is generally applicable to compounds subject to ABI in a wide range of concentrations. The model of Figure 8 is anticipated to be particularly relevant when the CAC is comparable or lower than the IC₅₀ value, a circumstance that is likely in the early phases of fragment-based lead discovery.

CONCLUSIONS

The effective use and reliable characterization of drug leads depends critically on their aggregation properties. The simplest approach is to determine the CAC of the inhibitor of interest and investigate its interactions with the target protein below the CAC value. However, this is not always possible because the CAC is often lower than or comparable to the K_d for specific binding, which means that interactions with the target protein need to be characterized at free ligand concentrations exceeding the CAC value, thus leading to aggregation and ABI. In this study, we propose a suite of integrated experiments to determine the mechanism of ABI and ABI-attenuation by agents such as the nonionic surfactant TX and the carrier protein HSA.

Our analyses have revealed two types of ligands that self-associate into aggregates with distinct morphologies and binding profiles. Type A aggregates define a new class of ligand assemblies that are inert and elicit only negligible nonspecific protein binding, while type B are invasive and adsorb target proteins causing nonspecific inhibition. While type B aggregation-based inhibitors lead to false positives, type A increase the likelihood of false negatives in drug screening. This is because type A aggregates still compete for specific binding, resulting in unusual bell-shaped titration patterns in which the fractional saturation of the target protein decreases with increasing inhibitor concentrations, when the total ligand concentration exceeds the CAC. Such artifacts can be identified and minimized through the

judicious use of TX and HSA, which are effective tools for diagnostic counter-screens. TX functions primarily by remodeling invasive type B aggregates into inert type A assemblies, while HSA acts as a sink for unbound inhibitors. However, TX and HSA often suppress both specific and nonspecific inhibitor-target protein interactions, and therefore reduction of false positives frequently occurs at the expense of introducing false negatives. This false positive vs false negative balance should be considered when utilizing attenuators of aggregation-based inhibition. Given the ubiquity of ABI, we anticipate that the ABI molecular mechanisms and resulting insight in ABI-attenuation provided here for ESIs will be transferable to other inhibitors as well.

EXPERIMENTAL PROCEDURES

Reagents and Sample Preparation.

The EPAC selective inhibitor (ESI) CE3F4R was generously provided by Dr. Frank Lezoualc'h (INSERM). Compound purities were greater than 95% as deemed by high-performance liquid-chromatography (HPLC). For ESI-09 purity of >99% was established through HPLC (t_R = 21.7 min), as previously described.⁷² For CE3F4R purity of >97% was established using supercritical fluid HPLC with a chiral stationary phase (t_R = 6.8 min). Triton X-100 (TX; product #T8787), cAMP, 8-anilinonaphthalene-1-sulfonic acid (ANS), 12C- and 13C-methyl labeled oleic acid, naproxen, ibuprofen, benzyl benzoate, miconazole, clotrimazole, nicardipine, and dansyl-arginine (DanR) were purchased from Sigma-Aldrich. Dansyl-phenylalanine (DanF) was purchased from Fisher Scientific. All reagents, with the exception of TX and cAMP, were prepared as 20–50 mM stock solutions in DMSO. Unless noted otherwise, all samples containing ESI-09 or CE3F4R contained 0.5% DMSO for every 100 μ M of ESI.

The cAMP-binding domain of hEPAC1 (residues 149–318) was prepared using a previously established protocol and is denoted here simply as EPAC1_{CBD}.^{31,57,73–76} Globulin and fatty acid free human serum albumin (rHSA) was purchased from Sigma-Aldrich (Sigma product no. A8763; 99% pure) and prepared in either NMR buffer (50 mM Tris, pH 7.6, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM DTT and 0.02% NaN₃) or sodium phosphate buffer (20 mM NaPO₄, pH 7.6, 50 mM NaCl in 99.9% D₂O). The concentrations of EPAC1_{CBD} and rHSA were determined by UV absorbance at 280 nm using extinction coefficients of 12 490 and 35 700 M⁻¹ cm⁻¹, respectively.^{65,77}

Dynamic Light Scattering.

Dynamic light scattering (DLS) measurements were acquired with a Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, U.K.) with a 4 mW He–Ne laser operating at a wavelength of 633 nm. Autocorrelation functions were accumulated for 150 s in replicates of 3–5 using a 173° scattering angle. The viscosity value for water was used in the analysis of all measurements. All samples were prepared in NMR buffer, prefiltered with 0.22 μ m filter and centrifuged for 5 min at 13 500 rpm prior to DLS measurements in disposable 40 μ L cuvettes (Malvern Instruments, ZEN0040).

Transmission Electron Microscopy.

Copper EM grids (400-mesh) freshly coated with a continuous layer of amorphous carbon were glow discharged with 5 mA for 15 s prior to sample addition. The ESIs, prepared as 3 μL aliquots in NMR buffer at 200 μM concentrations, were applied onto the EM grids, and excess sample was blotted with filter paper. The grids were then stained with 1% uranyl acetate for 30 s, dried under a lamp, loaded into a room temperature holder, and introduced into a JEOL 1200-EX electron microscope operated at 80 kV. All images were acquired on an AMT XR-41 Side-Mount Cooled 4 megapixel format CCD camera.

Surface Plasmon Resonance.

SPR experiments were performed on an OpenSPR instrument (Nicoya LifeSciences, Canada) using a nonfunctionalized gold nanoparticle sensor. All samples were prepared in NMR buffer and analyzed in duplicate. Samples were injected at a flow rate of 33 $\mu\text{L}/\text{min}$. Surface regeneration was not required for most samples based on the return to preinjection baseline, except for any injections involving HSA. 6 M guanidine HCl was injected for 2 min to remove bound HSA from the sensor. SPR data were processed and analyzed using TraceDrawer (Ridgeview Instruments AB).

NMR Spectroscopy.

NMR experiments were acquired with either a Bruker Avance 700 MHz spectrometer equipped with a 5 mm TCI cryoprobe or a Bruker Avance 850 MHz spectrometer equipped with a triple resonance TXI probe. All experiments were acquired at 306 K unless indicated otherwise. Multidimensional experiments were processed using the NMRpipe suite of programs and analyzed in SPARKY.^{78,79} One-dimensional experiments were processed and analyzed directly in Topspin. Additional details about data acquisition, processing, and analysis are described below.

Critical Aggregation Concentration (CAC) Determination of ESIs and TX.

Aggregate formation was probed using a combination of ^1H -1D Watergate and saturation transfer difference (STD) experiments. In the 1D Watergate experiments, the CAC of TX was measured by assessing changes in line widths at different concentrations of the molecule.^{17,41} The CAC was determined as the minimal concentration that increases peak width. However, for CE3F4R and ESI-09, there was no significant increase in apparent peak width over a wide range of concentrations. Hence, peak intensities were used as a proxy to determine the CAC of CE3F4R and ESI-09. Peak intensities are expected to increase proportionally with the compound's concentration. A deviation from this linear correlation between peak intensity and concentration, without significant changes in peak width, points to the compound being in a slow exchanging equilibrium between its monomeric and aggregate forms. If no new peaks arise as the concentration is increased past the CAC, as in the case of CE3F4R and ESI-09, this suggests that the invisible state is either lowly populated or very large. The former was ruled out because the peak intensity deviation from the linear correlation indicated there was a substantial concentration of the inhibitors that was unaccounted for. The CAC of the CE3F4R and ESI-09 inhibitors was thereby

determined by measuring the concentration at which the peak intensities deviated from the linear correlation.

STD NMR was used as an additional validation for the CAC determination of TX and the ESIs. A major determinant of the saturation transfer efficiency is the size of the molecules.⁴¹ Large molecules propagate saturation well because they exhibit effective cross-relaxation and have large networks of ^1H – ^1H dipolar couplings through which spin diffusion occurs effectively.⁴¹ In contrast, in small molecules (e.g., monomeric CE3F4R and ESI-09) cross-relaxation rates are slower and of opposite sign, which causes saturation to propagate inefficiently.⁴¹ However, when a small molecule binds to a large molecule, its cross-relaxation rates are transiently enhanced, and saturation can transfer effectively between the two molecules.⁴¹ Therefore, if a small molecule, alone in solution, was selectively irradiated at a specific resonance, saturation would only be expected to effectively transfer to its other resonances if it aggregated into a larger complex.

STD experiments were acquired as described previously^{31,80–82} using a selectively saturating train of 40 Gaussian-shaped pulses of 50 ms each. The STD spectrum was obtained by phase cycling subtraction of the on-resonance and off-resonance data in an interleaved manner. TX, CE3F4R, and ESI-09 were selectively irradiated at 7.48, 8.61, and 6.61 ppm, respectively, while the off-resonance control irradiation was at 30 ppm. The spectra were digitized with 32K complex points using a spectral width of 16 ppm centered at 4.7 ppm. Experiments were acquired with 256 scans, 16 dummy scans, and a recycle delay of 0.1 s preceding the Gaussian train for a total duration of around 15 min. Spectra were processed using an exponential multiplication window function with a 3 Hz line broadening.

Interaction of TX with Small Molecule Compounds.

Interactions between TX and other hydrophobic small molecules, such as the EPAC-selective inhibitors or aggregation-prone commercially available drugs (Figures S5A and S6A), were assessed through ^1H 1D line width and chemical shift analysis as discussed in the previous section. In addition, 2D NOESY and ROESY experiments were acquired for 100 μM ESI-09 with 0.025% TX and 500 μM CE3F4R in the presence of 0.05% TX. The NOESY and ROESY experiments each were digitized with 2K and 512 complex points in the direct and indirect dimensions, respectively. The spectral width in both dimensions was 12 ppm centered at 4.7 ppm. The NOESY was acquired with 64 scans, 64 dummy scans, a recycle delay of 1.25 s, and a mixing time of 250 ms for a total duration of 14.5 h. The ROESY experiment was acquired with 32 scans, 64 dummy scans, a recycle delay of 1.25 s, and a mixing time of 80 ms with a continuous wave spin-lock strength of 2.5 kHz. Intermolecular NOEs were normalized to the diagonal peak along the t_1 dimension.

Characterization of Specific and Nonspecific Interactions of ESIs with EPAC1_{CBD}.

Interactions of ESIs with EPAC1_{CBD} were assayed via $\{^{15}\text{N}$ – $^1\text{H}\}$ - heteronuclear single quantum coherence spectroscopy (HSQC). Spectra were acquired and processed as described previously.^{31,57,75,76,81,83} All spectra were shift referenced using ^{15}N -acetyl-glycine as an internal standard. Specific interactions of ESIs with EPAC1_{CBD} were measured by compounded chemical shift (CCS) differences calculated as follows:

$$\Delta\text{CCS} = \sqrt{(\delta_{\text{H1}} - \delta_{\text{H2}})^2 + (0.2 \times (\delta_{\text{N1}} - \delta_{\text{N2}}))^2} \quad (1)$$

The binding isotherms for CE3F4R were constructed by averaging the CCS of 35 residues, which had a CCS value greater than 0.025 ppm at any concentration in the titration. The same 35 residues were then used for the binding isotherm in the presence of HSA. The K_d and maximum CCS value (CCS_{max}) were fitted with the least-squares method assuming a 1:1 binding stoichiometry. The K_d was also fitted using a fixed CCS_{max} that was predicted previously (Figure 7B, black dashed curve).³¹ The apparent K_d values ($K_{d,\text{app}}$) in the presence of HSA were also computed either without imposing restrictions on the CCS_{max} (Figure 7B, red solid curve) or by using fixed CCS_{max} values determined from the fitting of the sample without HSA (Figure 7B, red dashed and dotted curves). For ESI-09, the binding isotherms were constructed similarly to how we described previously,³¹ with several residues being independently fitted using a 1:1 quadratic binding equation.

Nonspecific interactions of EPAC1_{CBD} with ESI aggregates were assessed by global losses in EPAC1_{CBD} HSQC peak intensities. The peak intensities of EPAC1_{CBD} in the presence of ESIs were normalized to a sample without ESI, and the average I/I_0 ratio of all residues were computed. If a peak intensity became unavailable at a given ESI concentration, the signal intensity at the peak position of the previous concentration was used. Errors on each I/I_0 ratio were calculated from propagation of the signal-to-noise ratios. The errors of all residues were then averaged to provide the error on the average I/I_0 values.

¹³C-Oleic Acid Assay.

Globulin and fatty acid free HSA (rHSA) was dissolved in sodium phosphate buffer to a concentration of 250 μM and incubated at 37 °C for 30 min. During this time, ¹³C-oleic acid (OA) was prepared as a 100 mM stock solution in DMSO-*d*₆ and incubated at 50 °C for 10 min. 1.5 mM OA was then added to the HSA sample, and it was further incubated at 37 °C for 2 h. {¹³C-¹H}-HSQC spectra were then acquired for the sample with and without ESIs. ¹³C OA acts as a probe for the different fatty acid and drug binding sites in HSA, as it results in a peak with a unique chemical shift for each site.^{25,63,64} When ligands outcompete OA at specific sites, those peaks will decrease in intensity or disappear altogether.^{25,63,64} {¹³C-¹H}-HSQC spectra were acquired with gradient and sensitivity enhancement, 64–128 scans, 16 dummy scans, a 1.4 s recycle delay, and spectral widths of 9765.625 (1H) and 5603.555 (13C) Hz digitized with 2K and 256 complex points, respectively. All experiments were performed at 298 K. The observed peaks were labeled as A–H, as described previously.^{25,63,64}

8-NBD-cAMP Fluorescence Competition Experiments.

Measurement of cAMP and ESI-09 binding affinities for EPAC1_{CBD} was achieved via competitive binding with the fluorescent cAMP analogue, 8-NBD-cAMP, using an approach similar to the ones described previously.^{55,56,84–86} The K_d of 8-NBD-cAMP for EPAC1_{CBD} was initially determined via a titration in which the 8-NBD-cAMP concentration was fixed at 250 nM, and EPAC1_{CBD} was added at concentrations ranging from 0.1 to 50 μM .

EPAC1_{CBD} and 8-NBD-cAMP concentrations were then fixed at 2.5 μ M and 250 nM, respectively, and cAMP or ESI-09 was added to outcompete 8-NBD-cAMP from EPAC1_{CBD}. Data were acquired with a BioTek Cytation 5 plate reader using Corning 96 well half area plates (Product No. 3881). The sample was excited at 485 nm, and emission was recorded at 535 nm. Competition curves were fitted using the approach described by Wang.⁸⁷

Fluorescence Competition Experiments for Dansylated Amino Acids with HSA.

The binding affinities of ESIs to HSA drug binding sites were determined with fluorescence competition experiments using dansylated phenylalanine (DanF) and arginine (DanR). DanR binds to HSA in a 1:1 stoichiometry selectively targeting Sudlow site 1,^{29,30,88} whereas DanF binds to both Sudlow sites 1 and 2.^{29,30,88} The binding affinities and stoichiometries of these ligands for HSA were determined using a previously described approach.^{29,30} All samples were prepared in 96-well plates and analyzed with a BioTek Cytation 5 plate reader using excitation and emission wavelengths of 370 and 460 nm for DanR and 370 and 480 nm for DanF. Competition experiments with ESIs were performed using 2.5 μ M HSA with 100 μ M of DanR or DanF. Since DanR has a 1:1 binding stoichiometry with HSA, the approach by Wang⁸⁷ was used to determine an apparent K_d for Sudlow site 1. For the DanF competition experiment, the average fractional saturation of HSA by DanF ($\langle v \rangle$) was determined as

$$\langle v \rangle = \frac{[PL] + 2[PL_2]}{[P] + [PL] + [PL_2] + [PI_a] + [PI_b] + [PI_{ab}]} \quad (2)$$

where P represents the concentration of free HSA, L represents the concentration of free DanF, and I represents the concentration of free ESI. The a and b subscripts for I reflect binding to either Sudlow sites 1 or 2. DanF binds both Sudlow sites with affinities that could not be resolved through a Scatchard analysis³⁰ (Figure S8). The equation can be rewritten in terms of the association constants:

$$\langle v \rangle = \frac{2K_L[L] + 2K_L^2[L]^2}{1 + 2K_L[L] + K_L^2[L]^2 + K_{i,a}[I] + K_{i,b}[I] + K_{i,a}K_{i,b}[I]^2} \quad (3)$$

where K_L is the association constant for DanF and $K_{i,a}$ and $K_{i,b}$ are the association constants for the ESI at sites a and b. A large excess of DanF was used for these experiments so it could be assumed that the free DanF concentration was roughly equivalent to the total DanF concentration. The total bound concentration of DanF, $[L]_B$, was then calculated by multiplying $\langle v \rangle$ by the total HSA concentration, $[P]_T$. To determine the total ESI concentration for fitting, we assumed that the free HSA concentration at all points throughout the titration was negligible. Therefore, the total bound ESI concentration, $[I]_B$, could be determined by

$$[I]_B = 2[P]_T - [L]_B \quad (4)$$

The total inhibitor concentration could then be determined by adding the total bound ESI concentration to the free ESI concentration. In the case of either the DanR or DanF competition experiments, the curves were fitted using the least-squares method assuming there were no additional binding sites on HSA for the ESIs. However, this is just an approximation as other experiments indicated there were additional binding sites for ESI-09. Hence, the affinities obtained with this method should be considered just as maximum apparent dissociation constants.

ANS Fluorescence Competition Experiments.

Additional binding of ESIs to HSA was probed with ANS fluorescence competition experiments. ANS has been reported to bind at four to five sites on HSA with low micromolar affinity.^{65,67,89} We followed a protocol similar to the experiments performed by Sudlow et al.^{65,90} to measure the binding stoichiometry and affinities for ANS to HSA under our experimental conditions. Competition experiments were performed with 2.5 μ M HSA with either 5 or 100 μ M ANS. ESI-09 or CE3F4R was then added over a range of concentrations to outcompete ANS. Measurements were recorded using an excitation wavelength of 360 nm and emission wavelengths of both 460 and 480 nm to check for blue shifts. The observed fluorescence changes (F_{obs}) were normalized to samples containing ANS alone (F_{ANS}) and ANS with HSA ($F_{\text{HSA:ANS}}$), which define the minimum and maximum observed ANS fluorescence, respectively:

$$\Delta F = \frac{F_{\text{obs}} - F_{\text{ANS}}}{F_{\text{HSA:ANS}} - F_{\text{ANS}}} \quad (5)$$

Supplementary Material

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ABBREVIATIONS USED

ABI	aggregation-based inhibition
BBR	base binding region
CAC	critical aggregation concentration
CBD	cAMP-binding domain
CMC	critical micelle concentration
CCS	compounded chemical shift

DLS	dynamic light scattering
EPAC	exchange protein directly activated by cAMP
ESI	EPAC-selective inhibitor
HSA	human serum albumin
OA	oleic acid
PBC	phosphate binding cassette
SPR	surface plasmon resonance
STD	saturation transfer difference
TX, TEM	transmission electron microscopy; Triton X-100

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Figure 1.

Evidence of CE3F4R and ESI-09 aggregation. (A) Molecular structure of CE3F4R. Hydrogens are labeled C1–C6 for NMR peak assignments. (B) DLS intensity profile of CE3F4R at 500 μM (2.5% DMSO). (C) EM images of CE3F4R aggregates ([CE3F4R] = 200 μM ; 1% DMSO). (D) Molecular structure of ESI-09. Hydrogens are labeled E1–E4 for NMR peak assignments. (E) DLS intensity profile of ESI-09 at 500 μM (2.5% DMSO). (F) EM images of ESI-09 aggregates ([ESI-09] = 200 μM). (G) Relative ^1H peak intensities of CE3F4R at different concentrations. The solid black line represents a linear extrapolation of the relative peak intensities based on the average of CE3F4R peaks at 50 μM . The vertical dashed line highlights the lowest CE3F4R concentration above which at least three peaks exhibit a significant deviation from the linearly projected relative peak intensity. The

asterisks mark the appearance of solid precipitate. (H) Saturation transfer reference (STR) (black) and STD spectra of CE3F4R at 100 (blue), 150 (green), and 200 (purple) μM . The peaks are assigned relative to the labeling in panel A. CE3F4R was saturated at the C1t resonance (sat. arrow). Additional arrows mark peaks arising from STDs. The # symbol marks a buffer impurity. (I) Similarly to panel G, the relative ^1H peak intensities of ESI-09 at different concentrations. (J) STD spectra of ESI-09 at different concentrations. ESI-09 was saturated at the E3 resonance (sat. arrow).

Figure 2.

Elucidation of specific vs nonspecific binding for EPAC-selective inhibitors. (A) Chemical shift changes of representative cAMP-bound EPAC1_{CBD} residues from the binding of CE3F4R (CE3) at concentrations ranging from 90 μ M (blue) to 3 mM (blue) ([EPAC1_{CBD}] = 150 μ M; [cAMP] = 1 mM). (B) Residue-specific CCS differences of EPAC1_{CBD} upon addition of 300 μ M (black) or 3 mM (blue) CE3F4R. (C) Chemical shift changes and intensity losses of representative EPAC1_{CBD} residues upon binding of ESI-09 at concentrations ranging from 50 to 250 μ M ([EPAC1_{CBD}] = 100 μ M). (D) Residue-specific CCS differences of 25 μ M EPAC1_{CBD} with 100 μ M ESI-09. (E) Binding isotherm for EPAC1_{CBD}:ESI-09 interactions measured through HSQC chemical shift perturbations. The different colored curves reflect the chemical shift changes of different residues. (F) K_d

measurements of cAMP (black) and ESI-09 (red) by fluorescence competition with 8-NBD-cAMP. (G) Average peak intensity changes of EPAC1_{CBD} vs. the concentration of CE3F4R (red), ESI-09 (blue), or DMSO (black). The DMSO curve reflects its concentration at the corresponding ESI concentrations. (H, I) Dynamic light scattering (DLS) volume distributions for EPAC1_{CBD} in the presence of CE3F4R (H) and ESI-09 (I), respectively. (J) Hypothetical thermodynamic cycle that describes both the specific 1:1 binding of an ESI with EPAC1_{CBD} as well as its development of large colloidal aggregates that can potentially interact with EPAC1_{CBD} nonspecifically.

Figure 3.

Evidence of coaggregation between TX and ESIs. (A) Region of TX ^1H spectra that shows concentration-dependent broadening and chemical shift changes of its aromatic resonances. The molecular structure of TX and its chemical shift assignments (T1–T7) are provided at the top. (B) TX line widths at half maximal peak intensity as a function of TX concentration. (C–D) ^1H spectra of CE3F4R (C) and ESI-09 (D) with and without TX at concentrations below their respective CACs. (E) Map of select intermolecular TX NOEs with CE3F4R and ESI-09. The relative NOE strength is depicted by the width of the red lines. (F) DLS intensity profile of 0.0075% TX in the presence of 100 μM (blue) or 200 μM (green) CE3F4R. The large error bars for the 100 μM CE3F4R sample are due to sample heterogeneity that changed with time, as illustrated in the individual intensity scattering

profiles in Figure S7A. The vertical dashed lines correspond to the central peak positions of TX micelles (black) and CE3F4R aggregates (red) in isolation. (G) Similar to panel F except for ESI-09.

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Figure 4.

Effect of TX on EPAC1_{CBD} interactions with CE3F4R and ESI-09. (A–B) Representative peaks from NH-HSQC spectra that demonstrate the effect of 0.1% TX on 150 μM EPAC1_{CBD} in complex with 1.5 mM cAMP and 300 μM CE3F4R. (C) Similar to panels A–B, but for 100 μM EPAC1_{CBD}:ESI-09 complex with 0.05% TX. (D) Representative peaks from NH-HSQC spectra of 100 μM EPAC1_{CBD} with 0.025% TX and various concentrations of ESI-09. The ESI-09 bound state without TX (green) is shown for chemical shift comparison. (E) The average normalized intensity ratios for the ESI-09 titration depicted in D (red) compared with intensity changes from DMSO (black) and ESI-09 alone (blue). (F) Changes in TX NMR peaks upon addition of ESI-09 (same samples as panels D, E). (G)

NH-HSQC comparison of signal loss from ESI-09 and partial regeneration from TX addition.

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Figure 5.

Characterization of ESI interactions with HSA. (A, B) ^1H proton peak intensities of CE3F4R (A) and ESI-09 (B) in the absence (black) and presence (red) of HSA. (C, D) ^1H - ^{13}C HSQC spectra of ^{13}C -oleic acid (OA) bound to HSA in the absence and presence of CE3F4R (C) ESI-09 (D). The peaks represent OA bound at different sites in HSA. Sites D and F correspond to Sudlow site's I and II (SS1 and SS2), respectively. (E) Competition of ESI-09 with DanR, which selectively binds Sudlow site I on HSA. (F) Competition of ESI-09 with DanF, which selectively binds Sudlow sites I and II on HSA. The reported dissociation constants for ESI-09 in panels E and F reflect an upper range that does not account for binding at other sites. (G) Scatchard plot for ANS binding to HSA. (H) Competition of ESI-09 with ANS for binding to HSA.

Figure 6.

Analysis of ESI aggregate disruption by HSA. (A–B) SPR response of CE3F4R (A) and ESI-09 (B) binding to a nonfunctionalized Au sensor at concentrations below (50 μM ; red) and above (250 μM ; blue/cyan) their CAC. The concentration of DMSO was 1.25% in both samples and the corresponding DMSO control (black/gray). (C) SPR of 250 μM ESI-09 in the presence of 25 μM HSA (blue/cyan). The corresponding reference of HSA with DMSO is shown in gray and black ($n = 2$). (D) Similar to panel C, but for 25 μM HSA with 500 μM ESI-09.

Figure 7.

Effect of HSA on ESI interactions with EPAC1_{CBD}. (A) Representative chemical shift changes for CE3F4R binding to EPAC1_{CBD} in the absence and presence of HSA. (B) Binding isotherms for CE3F4R binding to EPAC1_{CBD} in the absence (black) and presence (red) of HSA. The fitting of these curves was performed using a quadratic polynomial in which the K_d and maximum CCS value were simulated using a root-mean-square minimalization approach. Additional details of the fitting are explained in the Experimental Procedures. (C) Representative section of NH-HSQC that shows the chemical shift and intensity changes of EPAC1_{CBD} with ESI-09 and HSA. (D) Global average intensity changes in EPAC1_{CBD} HSQC peaks as a function of ESI-09 concentration. In the presence of HSA, the average I/I_0 of EPAC1_{CBD} with 100 μM ESI-09 is improved by 44%, and a

similar degree of signal loss is only reached around 400 μM ESI-09. (E) Chemical shift differences of EPAC1_{CBD} with ESI-09 in the absence and presence of HSA. The concentration of EPAC1_{CBD} in all samples is 100 μM , and the stoichiometric ratios of ESI-09 and HSA are listed in the plot. The average chemical shift change for all residues is provided in parentheses within the legend.

Figure 8.

Proposed mechanisms by which nonionic detergents (e.g., TX) and proteins (e.g., HSA) attenuate aggregation-based inhibition of specific targets (e.g., EPAC). Type A inhibitors generate inert aggregates with negligible adsorption of the target protein. Type B inhibitors generate invasive aggregates that adsorb and nonspecifically inhibit the target protein. Both types of aggregates result in a depletion of the specific inhibitory complex (ESI:EPAC₁_{CBD}), as illustrated in the bottom grid. For type A inhibitors the depletion of ESI:EPAC₁_{CBD} is due to the ESI aggregate acting as a sink for free ESI. In the case of type B inhibitors the depletion of ESI:EPAC₁_{CBD} is amplified as it is also caused by EPAC₁_{CBD} adsorption onto ESI aggregates. TX interacts with ESIs forming coaggregates, whose nature is dependent on the relative TX vs. ESI abundance. At high TX concentrations the coaggregates have TX-

like properties (i.e., inert/nonprotein binding, type A-like), but if the inhibitor is predominant, the coaggregates will have aggregation properties similar to that inhibitor (i.e., inert type A or invasive type B, depending on the inhibitor). In contrast, HSA binds the free inhibitor, reducing its free concentration and preventing it from aggregating. Both TX and HSA create binding equilibria that are competitive with respect to EPAC1_{CBD}. The predominant binding equilibrium of the free inhibitor (i.e., EPAC1_{CBD} vs aggregation vs TX/HSA) depends on the relative affinities in each pathway (i.e., $K_{d,EPAC}$ vs. $K_{d,agg}$ vs. $K_{d,TX}$ or $K_{d,HSA}$). n and m denote the stoichiometries of the EPAC1_{CBD}:ESI-aggregate and ESI:HSA complexes, respectively.