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# Broad-scale analysis of thermodynamic signatures in medicinal chemistry: are enthalpy-favored binders the better development option?

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**Thermodynamic profiles of ligand binding, particularly enthalpically favored binding signatures, have been suggested as a criterion to support the decision-making process around which compounds to select for further optimization in drug development. The concept was enthusiastically taken up, but turned out to be too superficial, either because many aspects determining thermodynamic profiles are insufficiently appreciated or because it is difficult to compare such data on a global scale. The impact of water, changes in protonation states, along with buffer dependencies and incompatible measurement conditions that are far from standard conditions hamper such broad-scale comparisons. However, thermodynamic signatures can make us aware of the impact of these aspects and provide important hints for improving our understanding of the binding process and defining criteria for drug optimization.**



## Thermodynamic signatures as a guideline for ligand optimization

Approximately 10 years ago, Ernesto Freire suggested consulting thermodynamic signatures to render promising small-molecule drug candidates superior with respect to further development, and showed that 'first in class' are not always 'best in class' [1]. This hypothesis was based on the assumption that enthalpically favored binders have a clear advantage over entropically favored binders, because the enthalpy term provides a measure of the number and strength of noncovalent interactions gained while moving from the solvated to protein-bound state [2]. In particular, the unfavorable desolvation of nonoptimally positioned polar groups of a ligand can result in an enthalpic penalty. In fact, medicinal chemists are often tempted to add an increasing number of hydrophobic groups to their initial lead scaffolds and to make development candidates more rigid to gain affinity, but this also enhances potency for entropic reasons (Fig. 1). As a disadvantage, the generated molecules are mostly compromised by low water solubility, increased toxicity because of unselective binding of the increasingly hydrophobic ligands, and reduced resistance tolerance.

The idea of favoring enthalpic binders was enthusiastically taken up by the medicinal chemistry community, because readily accessible and easily recordable parameters are much sought after for the support of the nontrivial decision over which molecules to take to the next level of development. This is a crucial decision with huge significance, because it involves large costs. Thus, many concepts have been suggested to allow the tailoring of molecular parameters that are important for the development along a more enthalpy rather than entropy-driven optimization strategy. Such ideas have also been extended toward increasingly smaller starting points for the de-

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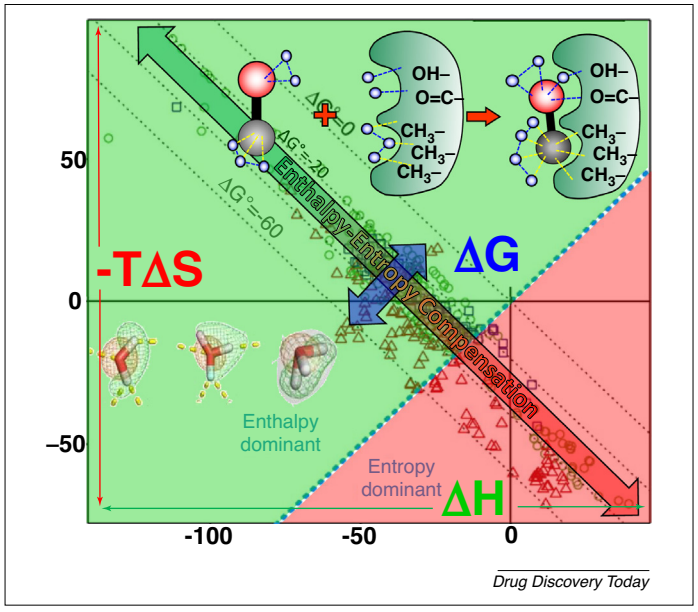


**FIGURE 1**

Thermodynamic data of experimentally studied protein–ligand complexes. The enthalpic contribution to binding ($\Delta H$) is depicted along the x-axis and the entropy portion ($-T\Delta S$) along the y-axis in kJ mol$^{-1}$. In the green area, the enthalpic portion dominates, whereas the entropic portion dominates in the red area. Along the main diagonal (blue arrow), $\Delta H$ and $-T\Delta S$ add and determine the Gibbs free energy of binding ($\Delta G$), leading to the small window of approximately $-15$ to $-60$ kJ mol$^{-1}$ accessible to medicinal chemists in their optimizations. Perpendicular along the secondary diagonal, both contributions work against each other and mutually compensate, resulting in a broad scatter of the data points. Here, the physically relevant enthalpy–entropy compensation superimposes with a mutual compensation originating from experimental inaccuracy. For example, any missing corrections of the measured data (e.g., unconsidered effects from superimposed protonation steps, concentration-dependent effects, or co-substrate binding, cf. Fig. 2 in the main text) will shift the mutual partitioning of a complex and the data will end up at another position in the diagram along the secondary diagonal. Data from protein–ligand complexes resulting from a medicinal chemistry optimization ($\Delta$) tend to increase in potency for entropic reasons in this diagram (lower right corner). At the top of the image, the protein–ligand binding process is schematically sketched, starting from separately solvated ligand and protein to form a complex, which is newly solvated. All steps contribute to the overall thermodynamic signature and the $\Delta H/-T\Delta S$ signature is particularly influenced by changes in the local water structure. On the left-hand side, the scatter density (from neutron diffraction) is shown around a water molecule in three different complexes. These data indicate that water molecules exhibit deviating rotational and translational degrees of freedom in different complexes and that they can vary among the complexes. This explains why water has such a strong impact on the thermodynamic profile in ligand binding. Adapted, with permission, from Ref. [11].

velopment and, thus, it was proposed that promising fragment hits should display enthalpically favored binding signatures [3–5].

An increasing amount of thermodynamic data from fully characterized case studies, mostly by means of isothermal titration calorimetry (ITC), have become available in easily searchable web-based databases, such as BindingDB (www.bindingdb.org/bind/index.jsp) [6–9], PDBcal [10], or SCORPIO (http://scorpio.biophysics.ismb.lon.ac.uk/scorpio.html) [11]. Therefore, large-scale analyses using statistical tools were performed to correlate chemical structure, or molecular properties derived thereof, with thermodynamic enthalpy–entropy profiles [5,11–13]. Even scoring functions to rank and validate computer-generated binding poses (e.g., from docking) were developed based on the partitioned enthalpy and entropy data stored in these databases. Furthermore, empirical scoring functions, based on a given master equation attempting to cover all contributing aspects that determine individual steps of the protein–ligand binding process, were trained with this information [14]. Following these concepts, the danger exists that only contributions that reflect common knowledge and agree with generally accepted models are considered in the training of such methods. However, this rarely leads to the new insights that are desperately needed. Indeed, most of the developed models focused on the direct interface between the bound ligand and the surrounding protein-binding pocket, because this information is intuitively visualized by protein crystallography or suggested by computer-modeling programs. Furthermore, this strategy is particularly tempting, because structure-based drug design and medicinal chemistry predominantly deal with the optimization of protein–ligand interactions established at a binding site.

Meanwhile, the initial enthusiasm around the use of thermodynamic data as an ultimate decision-making criterion was replaced by a more realistic view and we now appreciate such data as another source of information to learn about the properties of putative drug candidates [15]. However, the question remains: did the disillusion following the initial euphoria result from the fact that thermodynamic data were evaluated inadequately on the basis of incomplete information or incorrect assumptions? We easily tend to accept only correlations that reproduce well-accepted knowledge. It might be that affinity improvements for enthalpic reasons are an advantage for certain steps of the binding process; however, are they always the important and dominant ones and directly displayed in overall thermodynamic profiles? Here, I discuss three issues that underline why thermodynamic data are often inappropriately considered and misleadingly correlated.

## Thermodynamic signatures reflect the entire binding process, not only the protein–ligand interface

First, the thermodynamics of binding considers the entire binding process, from separated ligand and protein to the formed complex in aqueous solution, strictly speaking, in a buffered solution rich in ions and other components (Fig. 1). The multiple binding steps are dominated by the participation of water molecules, which impact both the enthalpic and entropic contributions of binding. Any change in the solvation structure along the path toward the formed complex influences the thermodynamic signature. In an ITC experiment, the gold standard for experimentally recording thermodynamic data, the balance sheet of the entire process is measured, comprising many small contributions. It is likely that several of them will cancel out or mutually compensate for each other. It can even happen that, overall, a step that does not correspond to the actual formation of the protein–ligand complex remains the dominating step. A case was recently reported in which the most flexible ligand in a congeneric series was, counterintuitively, entropically the most favored binder [16]. Upon fixation at the protein-binding site, a flexible ligand sacrifices a large number of internal rotational degrees of

freedom and, thus, experiences an entropic disadvantage. This also explains why correctly preorganized ligands exhibit an entropic advantage over more flexible analogs [17]. In the case of the counterintuitive entropic binding of the most flexible ligand to protein kinase A, experiments showed that the entropy benefit did not result from enhanced residual mobility of the protein or release of ordered water molecules to the bulk phase. Instead, the most flexible ligand adopted a highly organized, back-folded-on-itself conformation, which efficiently entraps a water molecule in aqueous solution before protein binding. Once the ligand is accommodated by the protein, the release of this fixed water molecule gives rise to the overwhelming entropic signature and explains the overall entropic advantage of this compound.

Furthermore, conformational and even configurational rearrangements of the protein upon binding of highly similar ligands can lead to large differences in the thermodynamic binding profiles. Such a dramatic enthalpy–entropy discrimination of nearly equipotent ligands was recently reported that differed solely by a single methyl group [18]. Responsible for this partitioning in a more enthalpic or entropic signature were, in this case, adaptations of the protein, which either remain almost unchanged or undergo huge conformational transformations upon ligand binding. Such changes are usually difficult to observe experimentally and most computational models will be unable to sample and, thus, predict such transformations. This either results from the fact that the required protein adaptations occur on a timescale which is usually difficult to access with standard molecular dynamics simulations or that such simulations are set up in a biased way, which, consequently, cannot sample such surprising effects across a set of highly congeneric ligands.

## Thermodynamic signatures are strongly modulated by the local water structure

Second, water displacement and entrapment at the binding site of a protein upon ligand binding and, thus, the burial of hydrophobic surfaces, is by no means only an entropy-driven process, as usually assumed by simple computer approaches. The entire range, from enthalpy-to-entropy domination, can be observed with respect to the thermodynamic profile and, surprisingly, often the enthalpic and entropic contributions mutually cancel out [16]. Therefore, changes in the water inventory stay unrecognized in the free energy balance. Given that most experiments record changes in the binding affinity (representing $\Delta G$), modulations of the water structure remain undetected. On first glance, this appears to have no consequence, because affinity is the main property to be optimized in drug development. However, if changes in the water structure are not considered (e.g. in computer approaches such as docking) the generated binding modes will usually be incorrect, even though the predicted affinities might agree well with the experiment. Nevertheless, incorrect binding modes are a serious problem, because subsequent attempts at structure-based ligand optimization will fail.

The broad scatter of contributions to the thermodynamic signature of water release and entrapment relates back to the ability of water to form sophisticated dynamic networks, which give rise to the structural and thermodynamic properties of water [19]. Each water molecule at the binding site will contribute differently to the thermodynamic signature, and this manifests once the water is released to the bulk phase upon ligand binding. Recent neutron diffraction studies disclosed how complex this situation can be, because the adopted water structure differs in its dynamic properties depending on the bound ligand (Fig. 1) [20]. Individual water molecules even change their rotational degrees of freedom depending on the type of bound ligand. These aspects will all influence the individual thermodynamic signature of a bound ligand in a distinct way, particularly because the contributions of water molecules to the overall thermodynamic partitioning are large. These contributions can easily range from 5 to 10 kJ/mol per water molecule [19].

Remarkably, water binding can also enhance ligand binding. As mentioned earlier, protein–ligand contacts mediated through water molecules usually do not contribute significantly to affinity. In cases where the interacting groups bear a charge, a net free energy contribution can remain [19]. However, much larger contributions occur when hydrophobic binding occurs in poorly or insufficiently solvated protein pockets [21]. In these, no price for desolvation is paid and, thus, a huge enthalpic advantage to the binding signature occurs. Steve Homans suggested that enthalpic ligand binding to the hydrophobic pocket of the mouse major urinary protein [22] results from a reduced water density in the pocket before ligand binding, thus explaining an enthalpy-dominated signature. It is likely that thermolysin is an extreme case with an almost completely unwettened pocket. Surprisingly, the newly formed solvation shell around a novel protein–ligand complex can also contribute to binding affinity. In addition to the region encompassing the binding site, the exposed portions of a bound ligand participate in the newly formed surface of the complex. Given that the water shell next to the complex surface can be highly structured, the quality and completeness of the water network formed across the new surface contributes to binding affinity [23]. Experimentally, the first and second layer of such water networks are only detectable in highly resolved crystal structures. Only then does the impact of these networks to binding affinity become obvious. In case of ligand portions extending beyond the flat and solvent-exposed S2′ pocket in thermolysin, optimization of such networks resulting in the formation of extensive circular water polygons improved ligand binding by up to a factor of 50, resulting in more enthalpic binding [23]. Recently, a similar effect was detected in a flat solvent-exposed pocket of a tRNA-binding protein, where the placement of methylated sugar-like substituents on the parent scaffold of an inhibitor enhanced binding [24]. Also here, the formation of extended water polygons across the newly created complex surface was initiated and contributed to binding affinity. Some care is needed when following the popular strategy of attaching terminal polar groups into the surface-exposed region opening to the surrounding solvent to overcome insufficient ligand solubility. A significant reduction in affinity results, because an enthalpic price for costly desolvation still has to be paid and the surface water network is perturbed in an unfavorable way [25].

## Partitioning of thermodynamic signatures varies with measurement conditions

Third, and likely the most fundamental aspect that makes the global analysis of thermodynamic signatures across multiple protein–ligand complexes questionable, arises from the fact that ITC measurements are conducted under different conditions using distinct concentrations, buffers, pH values, salts, other additives, and sometimes even different temperatures. This hampers a comparison of such data on an absolute scale [26]. In fact, thermodynamic measurements record data on a relative scale. To make these data widely comparable among many systems, they must be referred to standard conditions, which assume ideal solutions. However, in general, measurements are performed far from such ideal conditions. Thermodynamic signatures were systematically studied for selected protein–ligand complexes, with varying concentrations of both binding partners, while adding different salts, DMSO concentrations or other cryoprotectants [16,21,23,26]. Under these altered measuring

conditions, the free energy of binding remained almost unaffected, but large shifts were observed in the partitioning of enthalpy and entropy (Fig. 2). A striking example is ligand binding to protein kinase A in the presence or absence of an inhibitory peptide, mimicking the co-substrate, which did not affect the free energy. Across a congeneric ligand series, the relative amount of the partitioning of enthalpy and entropy also remained largely unchanged. However, the individual profiles of the various complexes displayed significantly different enthalpy–entropy partitioning on the absolute scale in the presence or absence of the peptide [16]. Furthermore, the binding event often occurs concomitantly with a change in protonation state. The release or entrapment of a proton by a titratable functional group of the ligand or the protein involves a heat signal, which adds to the signal of the binding process [28,29]. Such effects can be discovered by measuring the system in buffers with different heats of ionization. If such corrections are not applied or unwittingly ignored, the enthalpy of binding will be incorrect, and the partitioning of enthalpy and entropy will be wrong. In cases where the protonation change occurs remotely from the ligand-binding site and displays the same value across the studied congeneric ligand series, such corrections are not required. The heat effect will cancel out in a relative comparison of the thermodynamic data across the series. However, putting the data of such measured ligands on an absolute scale for a global comparison will be affected by the buffer used in the experiments. This means that, depending on the buffer chosen, absolute binding data can easily suggest either an enthalpy or entropy-dominated signature (Figs. 1 and 2). Without carefully reading the papers and analyzing the measurement protocols, thermodynamic data extracted from databases, such as BindingDB [6–9], PDBcal [10], or SCORPIO [11], and plugged into a correlation analysis will lead to false and misleading conclusions.



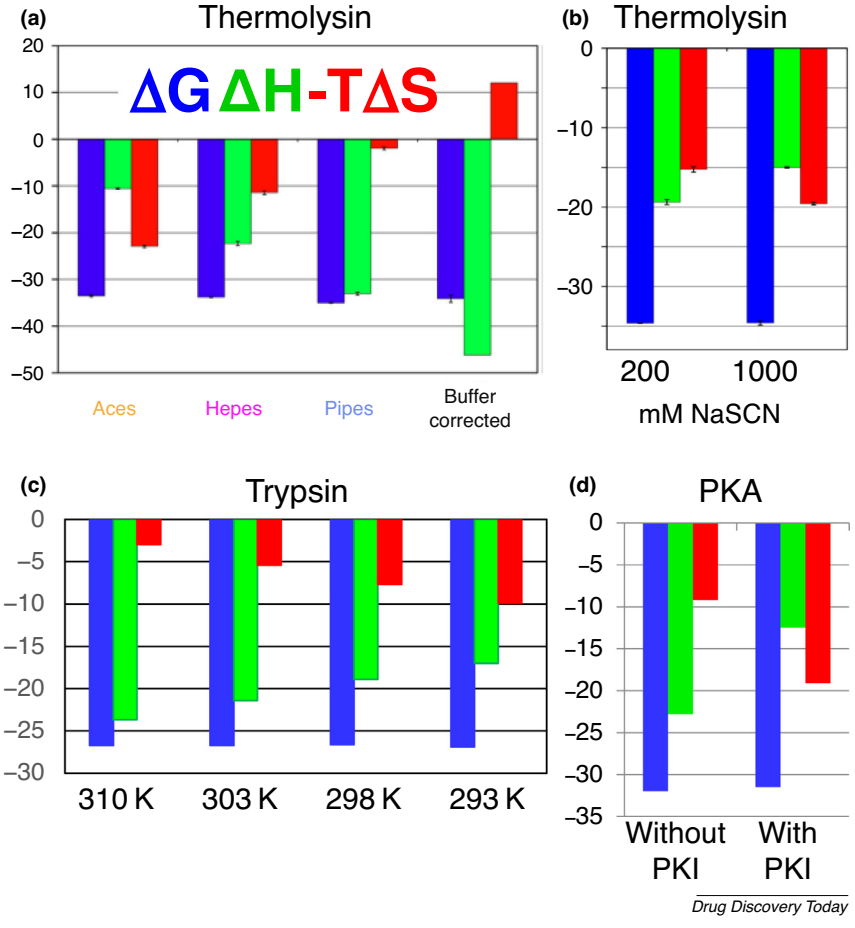


**FIGURE 2**

Thermodynamic signature determined by isothermal titration calorimetry (ITC; $\Delta G$, blue; $\Delta H$, green; $-T\Delta S$, red; all data in kJ mol$^{-1}$, mean error ~1 kJ mol$^{-1}$). Each diagram displays the binding signals of one ligand to a given protein recorded under different conditions. In all cases, the Gibbs free energy (blue column) remains almost unchanged. For all complexes, a high-resolution crystal structure has been determined and is referenced by the corresponding Protein Data Bank (PDB) code. (a) Binding of CbzGly$^{P}$Leu(*tert*-Butyl-Gly) to thermolysin (PDB: 5DPE) titrated from three buffers of deviating heat-of-ionization along with the buffer-corrected value [26]. A protonation step at a remote residue is superimposed, which does not affect $\Delta G$, but strongly shifts the $\Delta H/-T\Delta S$ partitioning. Depending which data are used to set up the correlation shown in Fig. 1 in the main text, the data points will end up at different positions along the secondary enthalpy–entropy compensation diagonal. (b) Binding of CbzGly$^{P}$Leu(*neo*-Pentyl-Gly) to thermolysin (PDB: 5DPF) titrated from Hepes buffer with deviating concentrations of added NaSCN (200 versus 1000 mM) [26]. (d) Titration of benzamidine to trypsin [27] at four temperatures (PDB: 5MO0/5MNG) [20]. Although $\Delta G$ remains unchanged, enthalpy and entropy modulate significantly with temperature. (d) Binding of an open-chain fasudil analog to protein kinase A (PKA; PBD: 5LCQ) with and without the bound co-substrate-analog inhibitor PKI [16]. Although this peptide does not interact directly with the ligand, $\Delta G$ remains unchanged, but enthalpy and entropy partition differently.

The phenomenon of enthalpy–entropy compensation has been discussed extensively in literature and multiple arguments for the existence of such an intrinsic and physically meaningful compensation in biological systems have been suggested [30–35]. However, superimposed on this intrinsic effect are experimental deficiencies and factors arising from the above-mentioned differences in the experimental setup that will result in apparent, but flawed enthalpy–entropy compensations [19,36]. Experiments only give access to $K_d$ and the heat of binding from which the Gibbs free energy and enthalpy are derived. Entropy is calculated as the numerical difference between the two properties, which automatically results in an enthalpy–entropy compensation. Therefore, any compensation arising from systematic errors in the determination of these quantities are difficult to separate from those representing a physically meaningful compensation. In a recent study, Bayesian statistics were applied to resolve the joint uncertainties in enthalpy and entropy, which is an essential prerequisite to evaluate any superimposed effects regarding enthalpy–entropy compensation [37]. One way to overcome this problem is to record thermodynamic data across congeneric series of ligands and to evaluate the data relative to each other. In this way, systematic errors and effects resulting from differences in the selected experimental conditions cancel each other out or do not affect the systematic trends of the thermodynamic data to be interpreted. It is likely that systematic errors cancel out better in such a series than noise, which will always be superimposed on any experimental technique. Therefore, an estimate of the accuracy of ITC experiments is essential [26,37]. As a direct consequence, we should avoid classifying ligands as enthalpy- or entropy-driven binders; in fact, we can only differentiate them as enthalpically or entropically more favored binders relative to one another.

## Way out of the dilemma: relative comparisons allow interpretation of thermodynamic signatures across congeneric ligand series

Accordingly, if, through broad-scale analyses by statistical means across multiple protein–ligand complexes on an absolute scale it is almost impossible to rank enthalpically favored binders as the better choice for optimization, what else can be learnt from the correlation of thermodynamic data in drug optimization? Data collected along with structural information across congeneric series while keeping the measurement conditions unchanged do provide information for parameterizing scoring functions (e.g., to assign values for the loss or gain of rotational degrees of freedom; the amount of surface areas buried upon binding; the contribution of individual hydrogen bonds; or the displacement of individual water molecules) [19,38]. Unfortunately, such systematic data are still rather scarce in literature and it is likely that they will depend on the studied system. To derive generally valid conclusions, the investigation of many such systems will be required. Luckily, a growing number of such studies can be observed in literature [39–42].

Yet, thermodynamic ITC data provide insights into additional, important aspects for drug optimization and rank the method as vital tool. The method allows researchers to record protonation-state changes of ligand and protein functional groups upon complex formation. Such shifts are crucial for binding, because they can transform a 'standard' hydrogen bond into a more strongly contributing 'charge assisted' one, particularly when a ligand changes from an uncharged state in solution or during membrane passage to a charged state in the protein binding pocket. This will boost affinity without the disadvantages of reduced bioavailability and paying the price for costly desolvation of a charged species. To achieve this goal, medicinal chemists have to correctly adjust the $pK_a$ values of their molecules, such that the desired protonation change only occurs upon protein binding.

ITC data can also help with the intriguing water issue. As mentioned earlier, water molecules mediating interactions between protein and ligand at the binding site often have minor impacts on the Gibbs free energy of binding because of extensive enthalpy–entropy compensation. This makes the detection of their involvement difficult [19]. It also explains why docking protocols neglecting the involvement of such interstitial water molecules can still produce reasonable affinity predictions, but these will be based on incorrect binding poses. If the water inventory is altered in congeneric ligand series, ITC usually detects huge changes in enthalpy–entropy partitioning. At the binding site, water molecules adopt deviating rotational states and can change with the accommodation of bound ligands (Fig. 1). Therefore, a large portion of the enthalpy–entropy inventory of the system is contained in the local dynamics of the water structure of each complex [20]. To correctly predict the thermodynamic properties and, above all, the binding free energy of protein–ligand complex formation, the behavior of the water molecules has to be described appropriately in, for example, computer simulations. Thermodynamic data, routinely recorded across series of ligands, can detect unexpected changes in thermodynamic signatures, originating from either changes in binding modes, protonation states, or water-mediated interactions. This provides essential information for the rational optimization of a drug candidate. Perhaps this, on first glance less 'hot tip' [2,15] stimulated by Freire's initial hypothesis [1] to consider thermodynamic signatures in ligand optimization for candidate selection, will turn out to provide highly valuable support to better understand and consider presently disregarded aspects of ligand binding in our optimizations. Based on this, the correlation of thermodynamic signatures across ligand series could positively impact our understanding of protein–ligand complex formation and drug optimization strategies in the future.

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