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Correcting CFTR folding defects by small-molecule correctors to cure cystic fibrosis

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Pharmacological intervention to treat the lethal genetic disease cystic fibrosis has become reality, even for the severe, most common folding mutant F508del CFTR. CFTR defects range from absence of the protein, misfolding that leads to degradation rather than cell-surface localization (such as F508del), to functional chloride-channel defects on the cell surface. Corrector and potentiator drugs improve cell-surface location and channel activity, respectively, and combination therapy of two correctors and a potentiator have shown synergy. Several combinations are in the drug-development pipeline and although the primary defect is not repaired, rescue levels are reaching those resembling a cure for CF. Combination therapy with correctors may also improve functional CFTR mutants and benefit patients on potentiator therapy.

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Introduction

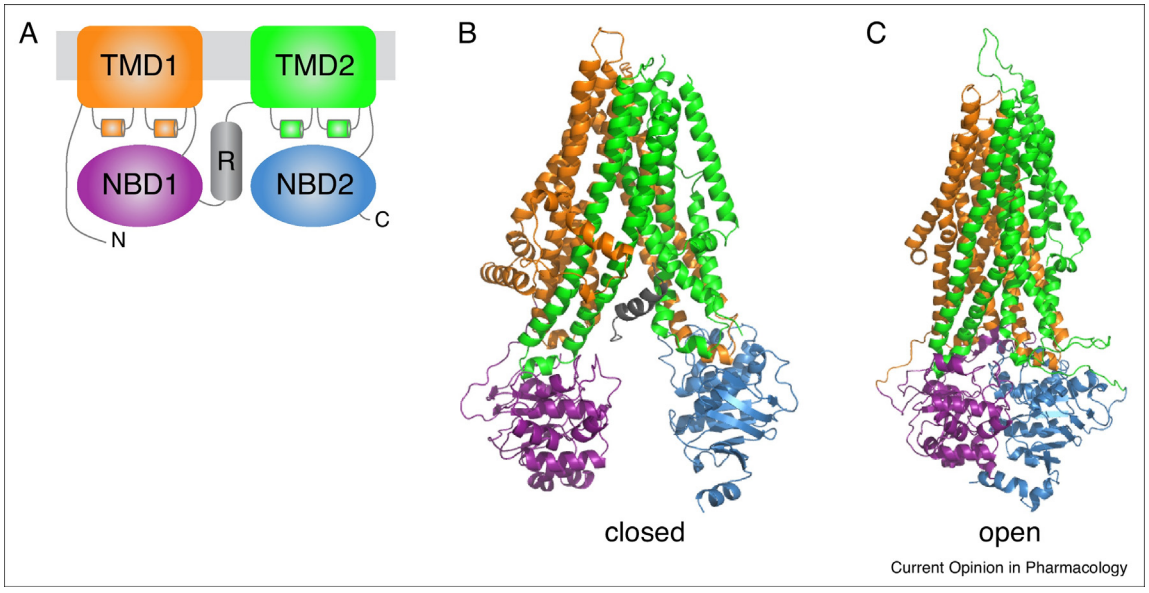
Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. CFTR is a chloride channel in the apical membrane of epithelial cells where it is essential for maintenance of salt and fluid homeostasis. The majority of disease-causing mutations lead to misfolding of CFTR, including the deletion of phenylalanine at position 508, F508del, occurring in ~90% of patient alleles [1]. Misfolding results in a defective channel and the mutant protein either is released to the cell surface, where it will display functional defects, or is retained in the ER and retrotranslocated into the cytosol for degradation by the proteasome [2]. Small-molecule correctors have been developed that rescue mutant CFTR from the ER; these are valuable drugs, which are supposed to rescue CFTR folding [3]. In this review we discuss what is required for correction of CFTR and how functional CFTR mutants may benefit from correctors. We review the most promising strategies that are being employed, and discuss why extant corrector drugs do not yet have what it takes to cure CF.

CFTR folding and misfolding

CFTR is an ABC transporter with two transmembrane domains (TMDs), two cytoplasmic nucleotide-binding domains (NBDs) and an instrinsically unstructured region (R) (Figure 1) that adapts its conformation upon binding NBD1, NBD2, the N-terminus of CFTR, the STAS domain of SLC26 transporters and probably other partners [4,5]. The TMDs form the channel across the membrane and the NBDs bind and hydrolyse ATP to stimulate opening and closing of the channel, regulated by (de)phosphorylation, which changes the interactions of R [6,7].

CFTR is synthesized at the ER membrane where the individual domains fold largely co-translationally into their three-dimensional structures [8]. The domains assemble and establish essential interdomain contacts, as evident from experiments and the various structures and structure models [9,10,11••] (Figure 1B and C). The intracellular loops (ICLs) that protrude from the TMDs into the cytoplasm form important contact sites that receive conformational cues from the NBDs and R to open and close the channel. Wild-type CFTR is transported to the plasma membrane, the apical cell surface, via the Golgi complex, where its glycans are modified into complex glycans [12]. Misfolded CFTR that is recognized as aberrant by the cell's quality control systems (e.g. F508del) is retained in the ER, retrotranslocated into the cytosol, and degraded by the proteasome [13,14]. Other misfolded, dysfunctional CFTR mutants are not recognized as aberrant and hence are transported to the cell surface with reduced or absent functionality. Examples are all CFTR mutants that cause disease but do leave the ER (e.g. G551D [1]). After all, some exceptions aside, a functional defect *has* to be derived from a conformational defect, whether recognized by chaperone machineries or not. Our unpublished analysis of CFTR2 mutants (Marcel van Willigen, BK, IB) has uncovered a

Figure 1



CFTR domain architecture. **(a)** Schematic model of CFTR. **(b)** Atomic structure of human CFTR in closed conformation (pdb 5uak), determined by cryo-EM [11••]. **(c)** Model of human CFTR in open conformation [9]. TMD1, orange; NBD1, purple; R, grey; TMD2, green; NBD2, skyblue.

wealth of folding defects in mutants that did leave the ER.

Complete correction would cure CF in patients with misfolding mutations. Correcting CFTR misfolding should allow the ER-retained mutant protein to be transported to the plasma membrane and may allow any mutant to gain functionality. Since virtually all disease-causing missense mutations misfold, these all may need or benefit from correctors to be rescued.

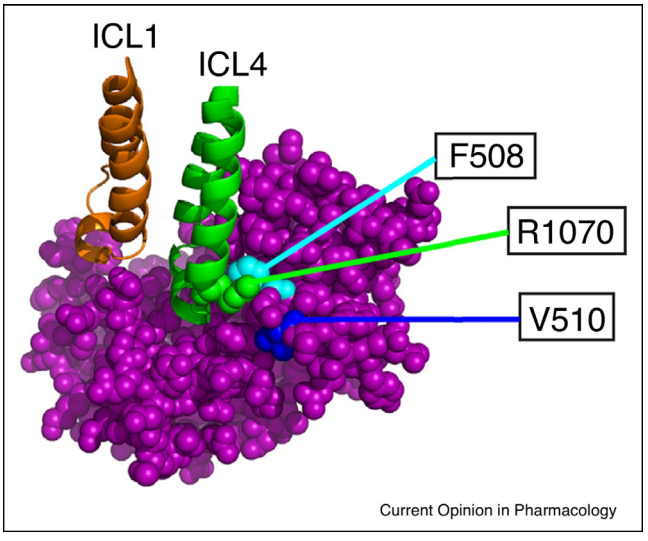
How to correct misfolded CFTR?

To bring functional CFTR to the cell surface two questions need to be answered: first, what does the CFTR molecule need to leave the ER and second, what does CFTR need to be a functional channel.

Manipulations of wild-type and mutant CFTR by temperature, compounds, or intragenic suppressor mutations have provided a wealth of information to confirm that many forms of dysfunctional CFTR are allowed to leave the ER. We will illustrate this below and will argue that individual compounds do not need to correct the primary defect nor channel function to be effective. The implications for therapy are hopeful.

Rescue efforts have focused on F508del CFTR, of which most is retained in the ER and degraded. A small fraction escapes ER quality control and is transported to the plasma membrane, but with a significantly reduced half-life at the cell surface compared to wild-type CFTR [15]. The identity of suppressor mutations might guide drug design. Peptides designed to mimic sorting motifs for example were shown to rescue F508del CFTR [16], and similarly, the synergy of various corrector drugs with I539T, the suppressor mutation that rescues NBD1 folding [17], instils confidence that a drug that repairs NBD1

Figure 2



F508 interacts with ICL4. Model of CFTR showing that F508 (cyan spheres) interacts with ICL4 in TMD2 (green). Suppressor mutants R1070W (R1070, green spheres) and V510D (V510, blue spheres) correct this interface when F508 is absent. NBD1, purple; ICL1, orange.

would be a beneficial addition to the (soon to be) available drugs.

Deletion of this phenylalanine (F508) in NBD1 causes not only NBD1 to misfold [17], but also causes domain-assembly defects [18–20]. F508 interacts with ICL4 in TMD2 (Figure 2) and its loss leaves a cavity and disrupts the NBD1–TMD2 interface critical for folding and function. The stability of the other domains is reduced as a result, underscoring the importance and sensitivity of CFTR's interdomain contacts. It is not enough to preserve the polypeptide backbone at position F508 for proper folding: specific side-chain interactions at this site are needed for CFTR domain assembly as well [21].

Despite the multiple defects in F508del CFTR, full rescue may be obtainable by rescue of the primary folding defect in NBD1. Indeed, combination of multiple stabilizing and second-site suppressor mutations in NBD1 restored F508del NBD1 folding (and likely its assembly with the other CFTR domains), resulting in rescue of CFTR to near wild-type levels, in terms of biosynthesis and release from the ER [17,22,23••,24,25••,26••], but at the expense of CFTR flexibility needed for proper channel kinetics [23••].

As the repair of a complete amino-acid deletion is not easy (in contrast, a gap left by only an absent side chain might be filled with a small molecule), many studies have focused on ways to rescue F508del CFTR from the ER. Not only the second-site suppressor mutations within NBD1, but also suppressor mutations that repair the NBD1-TMD2 interface (R1070W and V510D) (Figure 2) [25••,26••,27,28] and mutations in ER-exit [29] or retrieval motifs [30] have improved F508del CFTR expression at the cell surface, but only slightly so. Repair of both defects (NBD1 folding and domain assembly) or repair of more than one secondary defect beyond NBD1 shows synergy and improves F508del CFTR cell-surface expression and functionality up to wild-type levels [25••,26••].

The answer to the first question, what does the CFTR molecule need to leave the ER, appears to be: a folded NBD1 domain as well as assembled domains (Figure 3, path 1). But is this the correct answer? F508del CFTR retention in the ER is released to some extent already with either repair, and cell culture at reduced temperature (26 °C) results in increased cell surface expression [31]. At low temperature, global F508del CFTR conformation is somewhat improved [15,29] and the F508del CFTR channel is functional albeit with a low open probability [31]. Lowering the temperature improves CFTR conformation not only by thermodynamic stabilization but also by changes in cellular factors, because correction is not possible in every cell type [32]. Cellular proteostasis as well as the F508del CFTR interactome are different at low temperature [33••,34]. This includes differences in chaperone and co-chaperone associations and their kinetics. A striking case is the downregulation of Hsp90 co-chaperone Aha1, which improves F508del CFTR transport to the plasma membrane [35], establishing that F508del CFTR can be rescued by modifying proteostasis.

We conclude that the answer to the first question, is that to leave the ER, CFTR needs to be released by chaperones, and this can be accomplished by manipulating the interactome of CFTR, by rescuing NBD1 folding (Figure 3 path 2), or by rescuing domain assembly (Figure 3 path 3). From the many dysfunctional CFTR mutants at the cell surface we already know that release from the ER does not equate function. The implications have been shown to be hopeful for therapy, because multiple modes of rescue are possible and their combination often is synergistic (Figure 3 path 1 [25••,26••]).

Whereas suppressor mutations and temperature are therapeutically not applicable, large screens were set up by both academic and industrial groups to develop small molecules that improve CFTR folding and could be used as drugs. We here will not focus on the pioneering work by the Verkman lab [36], but rather on the more recent efforts that have been bringing drugs to the patient.

CFTR correctors

CFTR correctors are small molecules that make F508del CFTR leave the ER to improve cell-surface expression and optimally also increase gating and conductance. Correctors improve CFTR folding either by direct binding or by adapting protein homeostasis (proteostasis) and are respectively called pharmacological chaperones and proteostasis regulators. To date several compounds have been identified that improve CFTR export from the ER. Clinial-trial updates can be found at https://www.cff.org/Trials/pipeline.

The first correctors identified by high-throughput screening

The first two correctors identified by high-throughput screening (HTS) of a small-molecule library were bisaminomethylbithiazole C4 (Corr-4a) and quinazolinole C3 (VRT-325) [37,38]. Both ER and cell surface-localized F508del CFTR are stabilized by these compounds [37,38], which likely improve domain assembly [39,40] but do not or barely improve NBD1 stability [41–43]. Whereas C3 was reported to improve NBD1–TMD interactions, C4 acts primarily via the C-terminal half of CFTR [42,44]. Both compounds may not bind CFTR directly, as they are not specific for CFTR [38,45]. C3 and C4 did not make it to the clinic due to toxicity and low efficacy.

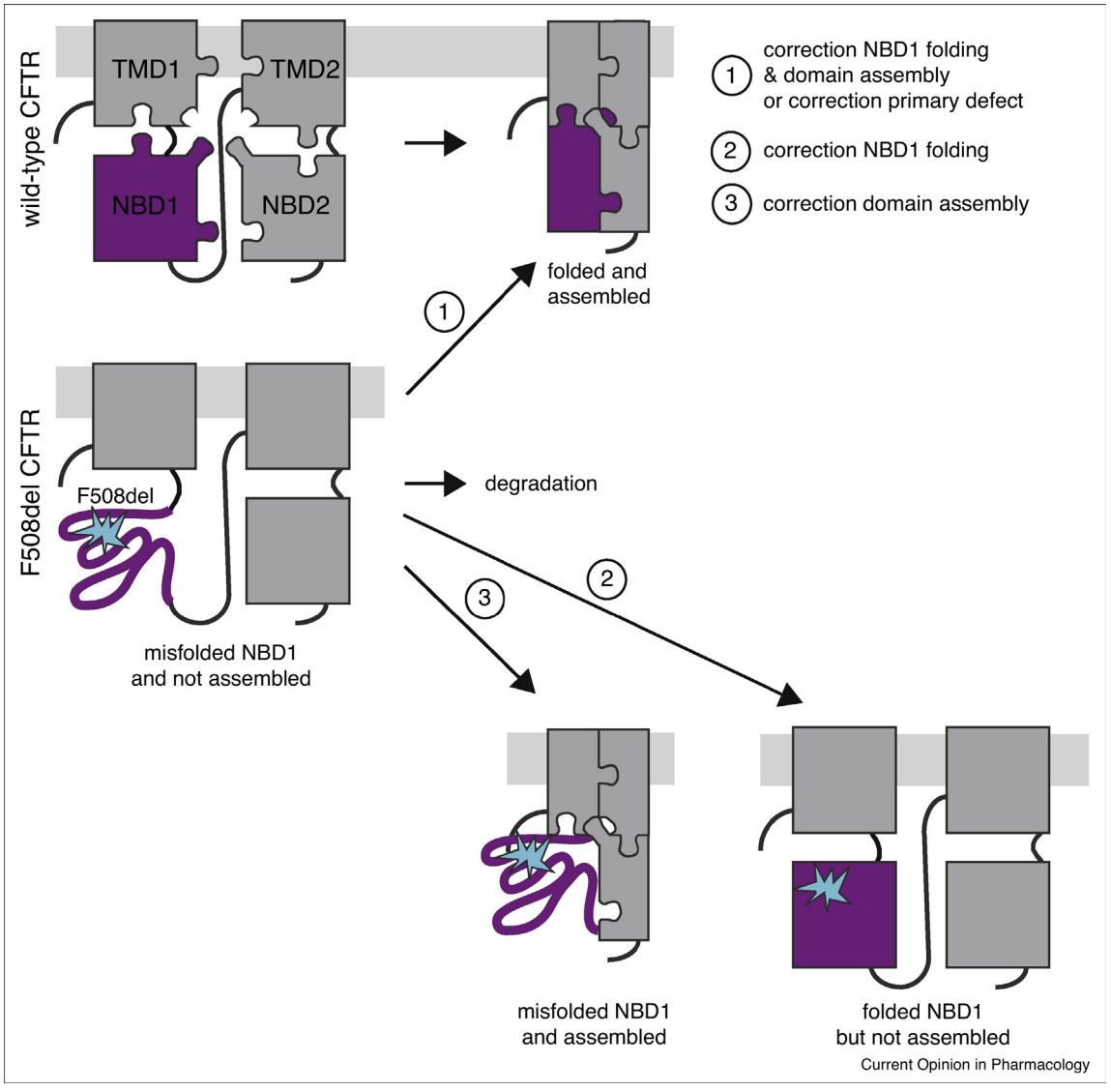
VX-809 and next generation correctors

Corrector VX-809 was developed by HTS and chemical optimization to become the first corrector approved for clinical use [45]. It corrects F508del CFTR with higher

potency and efficacy than C3 and C4, is more specific and is additive to C3 and C4, suggesting a different mode of action. VX-809-rescued total chloride transport in F508del CFTR HBE cells to ~14% of wild-type HBE cells [45]. VX-809 is suggested to bind CFTR directly as was shown in *in vitro* liposome studies (using VX-809 analog C18) [46] and by click-chemistry [47•], but the exact binding site remains unknown. VX-809 stabilizes TMD1 when expressed alone [42,48,49], improves TMD1 folding [48] and stabilizes interactions between the TMDs and NBDs [42,43,50•,51], but does not correct NBD1 [42,43]. While VX-809 has been found not to bind NBD1 [42], the VX-809 rescue mechanism appears to follow path 3 in Figure 3 by acting on TMD1.

Whereas VX-809 demonstrates proof of principle and signifies a thrilling development in CF, its modest efficacy triggered development of next-generation drugs. VX-661 is considered to be an improved VX-809 analog, and HTS in the presence of VX-809 or VX-661 [52] yielded correctors that were additive, leading to improved rescue. Those correctors, VX-440, VX-152 and VX-659, are currently in clinical trials [53]. CF now has become the first disease where multiple companies are developing

Figure 3



Different paths to correct CFTR folding. F508del CFTR folding can be corrected by rescuing either NBD1 folding or domain assembly. F508del CFTR folding will be best corrected by fully rescuing the primary defect in F508del-NBD1 or by both correcting NBD1 folding and domain assembly.

triple-combination drugs, combining 2 correctors with a potentiator.

Galapagos/AbbVie compounds

Four novel correctors in clinical trials have been developed by Galapagos/AbbVie using HTS. Correctors GLPG2222 and GLPG2851 (C1) are additive to GLPG2737 and GLPG3221 (C2) and may be combined in therapy. GLPG2222 shares structural similarities with VX-809 and VX-661 but is more potent [54]. Combinations of C1, C2 and a potentiator developed by Galapagos/AbbVie significantly increase chloride transport over Orkambi (VX-809 and VX-770) *in vitro*.

FDL169

The clinical candidate FDL169 stimulates F508del CFTR cell-surface expression with similar potency and efficacy as VX-809 but is not additive to VX-809 [55], suggesting the same mode of action. FDL169 is reported to have better drug properties such as a higher free fraction in human serum and improved distribution in the lung [55,56]. FDL169 may be an alternative to VX-809 as a therapy.

Cavosonstat and Riociguat

Cavosonstat (N91115) and Riociguat are novel CFTR correctors in clinical trials that act on components of the CFTR interactome, and thus can be considered as proteostasis regulators. While Riociguat increases sensitivity of soluble guanylate cyclase (sGC) to NO, Cavosonstat inhibits S-nitrosoglutathione reductase (GSNOR), which increases GSNO and NO levels that are lowered in CF tissue [57]. This leads to a postulated chaperone-dependent increase of CFTR levels, stability and function. Because of their indirect action off-target effects were expected, but so far they have been tolerated well [58].

Amplifier PTI-428

Unlike proteostasis regulators that indirectly improve CFTR folding, amplifiers are compounds that increase CFTR expression and thus increase the protein load in the ER. To correct CFTR function, these should always be combined with correctors and potentiators. A small-molecule HDAC7 inhibitor SAHA1 not only amplifies F508del-CFTR expression, but also promotes significant transport of F508del-CFTR to the cell surface [59] by reshaping CFTR's proteostasis network [33••]. These studies set the stage for development of a specific CFTR amplifier PTI-428 that increases the CFTR pool for correctors to act upon. Possible mode of action of this novel amplifier involves improving mRNA stability and/or events surrounding CFTR translation.

The future

Despite the large efforts to find potent CFTR correctors, the rescue obtained by individual correctors has been limited, because F508del CFTR suffers from multiple defects that each need correction [25••,26••]. None of the correctors developed so far completely corrects the primary F508del CFTR defect; they appear to primarily improve domain assembly. The combination of corrector and potentiator activity in a single molecule has not yielded competitive efficacy over the single correctors either [60].

At this point the favorite strategy is to combine compounds with different modes of action that together correct multiple folding defects. This includes combinations of drugs that increase CFTR quantity, improve transport from ER to the cell surface, improve folding/conformation, and drugs that improve CFTR channel function (called potentiators). The compounds that bind directly to CFTR are presumed to have fewer off-target effects than proteostasis regulators but the combination may be key. The near future will see a number of these drug combinations approved for CF, with the expectation that significant rescue, close to a cure for CF, will be obtained. Unfortunately, patients with CFTR variants that do leave the ER are not considered to benefit from combination therapy and are tested with potentiators only. We would argue that combination therapy with a corrector may benefit some of these patients as well.

The question remains whether a single compound will ever be able to restore the primary defect. Some dual-acting compounds have been identified [61,62], but they have not beaten the drug combinations. Perhaps not for F508del CFTR because of its deletion in the polypeptide backbone, but it may be different for other missense mutations. Over 2000 CF-causing mutations have been identified in CFTR, of which an increasing number have been demonstrated to cause CF. These usually have different primary folding defects, which in many cases results in similar overall misfolding and domain assembly defects. While correction of the primary defect would probably be best, most of the general misfolding defects are rescued by the same correctors. VX-809 has been shown *in vitro* to correct many more mutants than F508del alone [48,51], and C4 even works better on mutant V232D than on F508del [44]. As the potentiator Kalydeco (VX-770) was FDA approved for 33 rare mutations, with the majority based on *in vitro* data, there is hope that the patient pool benefiting from the CF clinical pipeline will expand significantly in the near future. Knowing exactly how the mutations disturb folding and the modes of action of drugs will help predict which correctors can rescue which mutants.

Despite all efforts, there still will be patients with CFTR variants that are not rescued by any of the drugs in the development pipeline and for those specific drug screens would be needed. Based on thorough conformational analysis of each mutant, a screen for repair of the primary defect may be an option, but a more rapid and practical

approach would be to simply repeat high-throughput screens with existing compound libraries on these difficult variants. The read-out of (functional) CFTR at the cell surface has shown to yield most of the drugs in the pipeline right now for F508del CFTR, and for FDA approval mode of action is not relevant. Doing such a screen with all variants that cannot be rescued with present correctors may yield novel compounds that act on multiple mutants, which would lower the cost of development. Conformational theratyping analysis will provide information on whether existing compounds act on a mutant, even in the absence of rescue. Such a compound may be added to the screen to increase chances of success, as was done for the development of several second-generation correctors.

Conclusion

The efficacy of individual current correctors is low because they do not fix the primary folding defects of the misfolded mutant CFTR (Figure 3). Combining correctors with different modes of action has been shown to improve efficacy and is likely to achieve rescue resembling a cure for cystic fibrosis. CFTR variants with functional defects should be considered for combination therapy with correctors as well.

Conflict of interest statement

IB declares no conflict of interest, but discloses collaborations with Galapagos and Vertex Pharmaceuticals.

Acknowledgements

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- • of special interest
- •• of outstanding interest

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