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Deciphering the Nonsense Readthrough Mechanism of Action of Ataluren: An *in Silico* Compared Study

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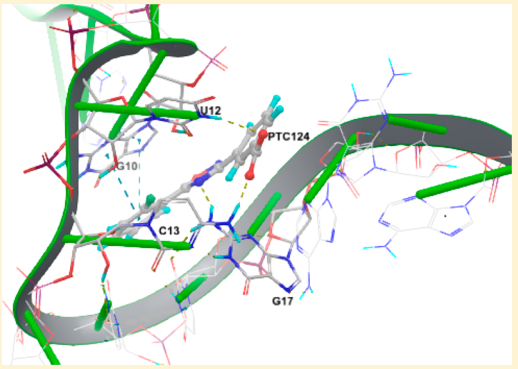
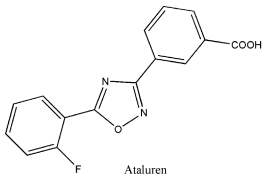
Supporting Information

ABSTRACT: Ataluren was reported to suppress nonsense mutations by promoting the readthrough of premature stop codons, although its mechanism of action (MOA) is still debated. The likely interaction of Ataluren with CFTR-mRNA has been previously studied by molecular dynamics. In this work we extended the modeling of Ataluren's MOA by complementary computational approaches such as induced fit docking (IFD), quantum polarized ligand docking (QPLD), MM-GBSA free-energy calculations, and computational mutagenesis. In addition to CFTR-mRNA, this study considered other model targets implicated in the translation process, such as eukaryotic rRNA 18S, prokaryotic rRNA 16S, and eukaryotic Release Factor 1 (eRF1), and we performed a comparison with a new promising Ataluren analogue (NV2445) and with a series of aminoglycosides, known to suppress the normal proofreading function of the ribosome. Results confirmed mRNA as the most likely candidate target for Ataluren and its analogue, and binding energies calculated after computational mutagenesis highlighted how Ataluren's interaction with the premature stop codon could be affected by ancillary nucleotides in the genetic context.

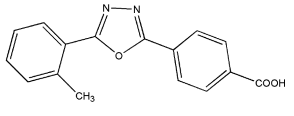


KEYWORDS: *Induced fit docking, QPLD, MM-GBSA, computational mutagenesis, oxadiazoles, premature termination codons*

A nonsense mutation is a point nucleotide change in a DNA sequence that introduces a premature termination codon (PTC) in the mRNA sequence encoding a functional protein. The UGA, UAG, and UAA PTCs cause inappropriate termination of the translation.[1] Nonsense mutations are responsible for about 12% of human inherited diseases,[2] including cystic fibrosis (CF).[3−6] In the case of CF, about 10% of the patients possess a PTC in their CF transmembrane regulator gene (CFTR). These PTCs lead to the premature termination of translation so producing truncated proteins.[3] The lack of adequate levels of the CFTR, the chloride channel required for the regular function of different organs, is responsible for a more severe form of CF disease.[7] The therapeutic approach to CF is mainly symptomatic, and only in the past decade a pharmaceutical approach has been proposed to rescue the protein production by selective translational readthrough of the PTC. To this aim, aminoglycosides (e.g., tobramycin, paromomycin, etc.) are known to possess the ability to readthrough stop codons. However, their lack of selectivity results in the undesired readthrough of correctly positioned stop codons, originating toxic aggregates that may cause ototoxicity and nephrotoxicity. In this context, Ataluren, also known as PTC124, is a 5-(fluorophenyl)-1,2,4-oxadiazolyl-benzoic acid which was launched by PTC-Therapeutics to promote the readthrough of premature but not normal termination codons in HEK293 cells.[8] It is less toxic than aminoglycosides and has been suggested as a potential drug for the treatment of genetic disorders caused by nonsense mutations, particularly those involving the UGA PTC.[9] Ataluren was originally selected by high throughput screening based on firefly luciferase (FLuc) reporter assays.[8] Its readthrough activity was confirmed by using orthogonal assays with two different reporters: H2B-GFP-opal Luc190.[10]



Ataluren



NV2445

Phase II and III clinical trials showed an improvement in markers of CFTR function but no improvements in sweat

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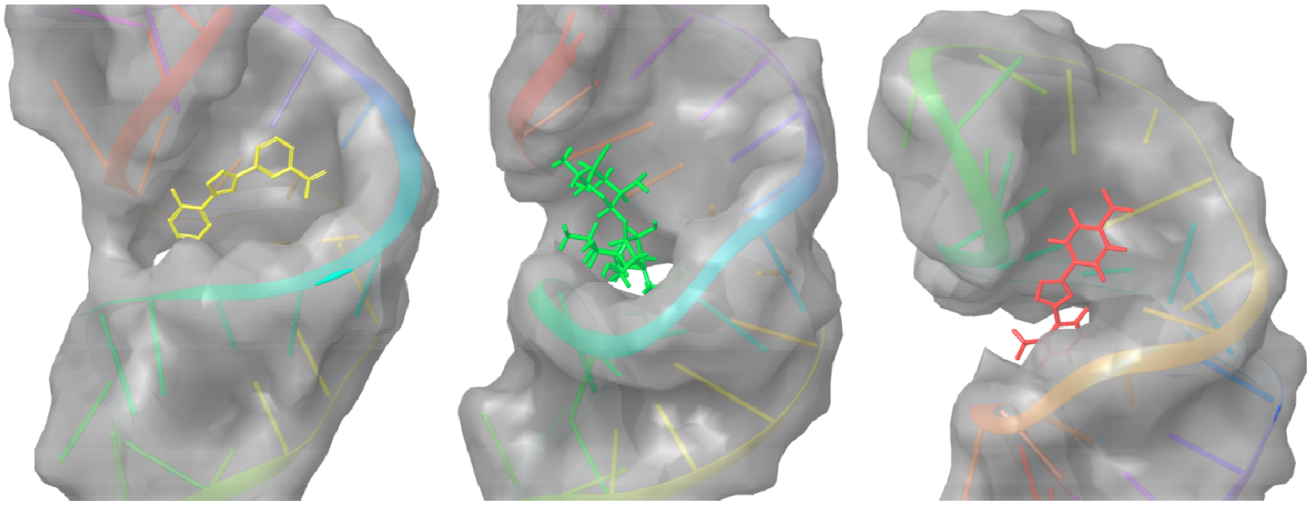

Figure 1. Binding poses of Ataluren (yellow), paromomycin, PDB ID: 1PBR (green), and NV2445 (red) into the rRNA 16S.

chloride levels or nasal potential difference.[11] Phase III Clinical trials for CF patients evaluated the long-term safety of PTC124 and lead to the hypothesis that Ataluren and tobramycin are in competition with each other, by acting at the level of the ribosome. Today, Ataluren is among the lead-like translational readthrough inducing drugs (TRIDs) despite the fact that its real efficacy in CF patients and its effective biological target are still not clear, encouraging research in this field.[12]

In this context, *in silico* studies, widely used to screen potential drugs for a given target, can be inversely used to screen targets for a given drug. A previous computational approach from our group has proposed mRNA as a putative target for Ataluren and provided an interpretation of Ataluren's specificity/selectivity toward a given UGA stop codon.[10] Indeed, Ataluren interacts differently with nonsense mutations of the CFTR gene such as G542X, R1162X, and W1282X, depending on the genetic context, since the sequence of nucleotides upstream and downstream of the PTC are involved even in the basal translational readthrough process.[13,14] These considerations explain the debate about the efficacy of Ataluren in CF therapy and urge the need for further investigations on its MOA and the importance of the research of new TRIDs.[15−17] Recent papers by Roy et al. showed that PTC124 has a selectivity for the ribosomal A site and that it promotes insertion of near cognate tRNAs at the PTC site.[18] Such suggested interaction of PTC124 with the ribosome was further supported by the finding that Tobramycin is a strong inhibitor of PTC124, probably by competition for the ribosomal A site.[19]

On these bases, we decided to combine different computational approaches for comparing different drug−target interactions to unravel the most likely MOA of Ataluren. In this work, we investigated interactions of Ataluren with a number of targets implicated in the translation of the message deriving from the gene itself: bacterial rRNA 16S and human rRNA 18S, which are proven targets for most common aminoglycoside TRIDs; eukaryotic release factor eRF1 to assess Ataluren's potential influence onto the ending of the translation process; human CFTR-mRNA fragments to evaluate the binding energy as a function of virtually introduced mutations. With respect to previous computational approaches that were considering Ataluren in its undissociated form,[10] we take into account the role of physiological pH which could determine ambiguous poses and interactions during the simulation. Modeling studies were performed by previously optimizing structures at pH = 7.2 ± 0.2 (SI), thus resulting in studying target's interactions with Ataluren in its dissociated form.

The starting hypothesis is based on the fact that Ataluren, possessing readthrough activity like aminoglycosides, could carry out its action interacting at the level of the ribosome. It is known that aminoglycosides exert their antibacterial action interacting at the rRNA 16S level of the prokaryotic center of decoding, located in the 30S subunit. Given the claimed parallelism between Ataluren and aminoglycosides,[8] we explored the potential interaction of Ataluren with rRNA 16S by using induced fit docking (IFD) and quantum polarized ligand docking (QPLD) and compared it with the known capability of aminoglycosides to interact with the same target. (PDB ID: 1PBR) Therefore, in addition to IFD, we used the QPLD protocol,[20] which uses quantum mechanical calculations in place of the force field to generate partial charges on the ligand atoms in the field of the receptor. This analysis was also carried out for some recently reported Ataluren analogues showing readthrough activity (SI)[16] and a new promising Ataluren's analogue NV2445, which was recently shown to rescue the CFTR functionality.[17]

Qualitatively, a visual comparison between the best poses of Ataluren, NV2445, and paromomycin in the pocket of rRNA 16S is suggesting that Ataluren and NV2445 do not fit as well as the model aminoglycoside in the binding site (Figure 1). Quantitatively, the obtained binding energies data showed that the affinity of Ataluren and its analogues toward the prokaryotic rRNA 16S target was much lower compared to a series of aminoglycosides (See Table 1 in SI), in agreement with the reported lack of Ataluren's antibacterial activity.[11] Additionally, since aminoglycosides are able to induce misreading also in eukaryotes,[21] a similar study has been conducted by IFD and QPLD docking to compare the affinity of Ataluren and its analogues toward the rRNA 18S ribosomal subunit. The structure of rRNA 18S bound to paromomycin obtained by means of NMR was used as template (PDB ID: 1FYP) for PTC124 and NV2445's best poses generated with IFD, as shown in Figure 2. The PTC124/rRNA18S binding energy (ΔG_{bind}) was estimated to be −7.3 kcal/mol (by IFD) or +0.1 kcal/mol (by QPLD), clearly demonstrating a much

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Table 1. $\Delta G_{binding}$ and Docking Score Values for the Poses of the Complexes between Ataluren and 12 Virtually Mutated mRNAs, Generated by IFD e MM-GBSA Compared to Values Obtained for Not Virtually Mutated CFTR^{opal}-mRNA Fragment Containing the Pathological UGA PTC

Target mRNA fragment		ΔGbind (kcal/mol)	Docking score
Unchanged CFTR^{opal}mRNA		−21.257	−6.920
Virtually mutated CFTRopal mRNA			
Susbtituted	Inserted		
G10	A	−10.296	−6.242
	C	−8.114	−5.307
	U	−5.988	−5.432
G19	A	−17.020	−4.822
	C	−19.692	−5.092
	U	−17.925	−5.550
U12	A	−17.464	−4.510
	C	−15.757	−5.426
	G	−16.789	−6.316
C13	**A**	−13.246	−5.251
	G	−15.068	−4.288
	U	−14.854	−5.503

lower affinity compared to paromomycin/rRNA18S for which ΔG_{bind} = −106.5 kcal/mol (by IFD) or ΔG_{bind} = −91.8 kcal/mol (by QPLD) was calculated. A similar situation was found considering other aminoglycosides, NV2445, and other Ataluren's analogues (See Table 1 in the SI). Based on the low affinity of Ataluren toward the ribosomal rRNA 18S region, we decided to explore release factors as an alternative target. In fact, in eukaryotic protein synthesis, the recognition of the stop codons, and therefore the termination of the translation process, is carried out by the release factor eRF1 (PDB ID: 2LLX). In light of this information, IFD simulations were conducted centering the potential grid on residues Lys63, Gly31, and Val101 (see SI). The UGA triplet was used as reference ligand since such stop codon is the natural target for eRF1. Indeed, the calculated binding energies showed that UGA/eRF1 interaction ($\Delta G_{binding}$= −9.2 kcal/mol) was more favored than both Ataluren/eRF1 and NV2445/eRF1 ($\Delta G_{binding}$ = −3.3 kcal/mol and −3.4 kcal/mol, respectively)) as shown also by comparison between the best IFD pose for Ataluren, its analogue NV2445, and the UGA stop codon in the eRF1 binding site (see Figure 3 and SI). Since interactions with rRNA and release factors would have affected also the normal translation of proteins, the low affinity of PTC124 for rRNA and eRF1 agrees with the absence of side effects and with Ataluren's selectivity in the readthrough of PTCs. In the light of these results obtained by means of different computational techniques, the mRNA bearing the PTC UGA still remains the most likely target of Ataluren and NV2445. This was confirmed by IFD docking studies performed using a 33-codon mRNA fragment bearing an UGA PTC (at positions U16-G17-A18 of the fragment), mimicking the mRNA resulting from the G542X mutation in the CFTR gene. Ataluren's simulation was started at the shortest interaction distance pose resulting from the equilibrium of previous molecular dynamics studies.[10]

IFD run generated poses of Ataluren rotated about 180° with respect to the carboxyl group and confirmed the $\pi-\pi$ stacking interaction as the preponderant driving force in the complex stabilization due to the planar and aromatic structure of Ataluren. Additionally, hydrogen bonds through the ionized carboxylic group have been identified to occur with uracil (U12 in the fragment) at position −4 and cytosine (C13 in the fragment) at position −3 with respect to the PTC, numbered according to the labeling of nucleotides surrounding a stop codon.[13] The best IFD pose is illustrated in Figure 4 and had favorable docking score values (See SI). The reliability of the docking results, obtained *in vacuo*, was checked by calculating the ΔG_{bind} of the Ataluren−mRNA complex by means of the MM-GBSA approach. The ΔG_{bind} are similar (ΔG_{bind}= −21.26 kcal/mol by IFD; ΔG_{bind}= −22.05 kcal/mol by QPLD). Interestingly, both results obtained from previously reported dynamics simulations and hereby docking studies performed at different levels of accuracy highlighted interactions with nucleotides neighboring the PTC. Moreover, experimental studies on Ataluren have suggested the involvement of the "fourth base" (i.e., the nucleotide at position +1 with respect to the PTC), in the observed readthrough selectivity, raising the hypothesis that the specificity of G542X readthrough could be sequence specific and not related only to the UGA PTC.

Therefore, we decided to quantify the effect of neighboring bases on the affinity between Ataluren and the UGA PTC in the nonsense mutated CFTR-mRNA fragment. The evaluation of sequence specificity in the Ataluren/UGA interaction was carried out by computational mutagenesis, by introducing alternative nucleotides replacing the nucleotides most involved in the interaction with Ataluren: G at positions −6 (G10 in the fragment) and +1 (the "fourth base" G19 in the fragment), C at position −3 (C13 in the fragment), as well as U at position −4 (U12 in the fragment) with respect to the UGA PTC. Each

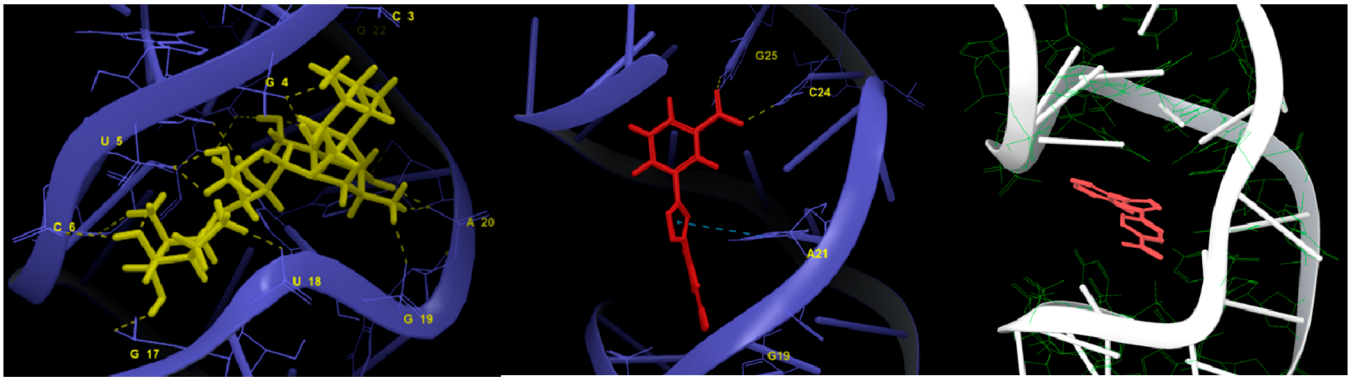

Figure 2. Paromomycin bound to rRNA 18S (PDB ID:1FYP) (left); Ataluren (middle); and NV2445 (right) bound to rRNA18S.

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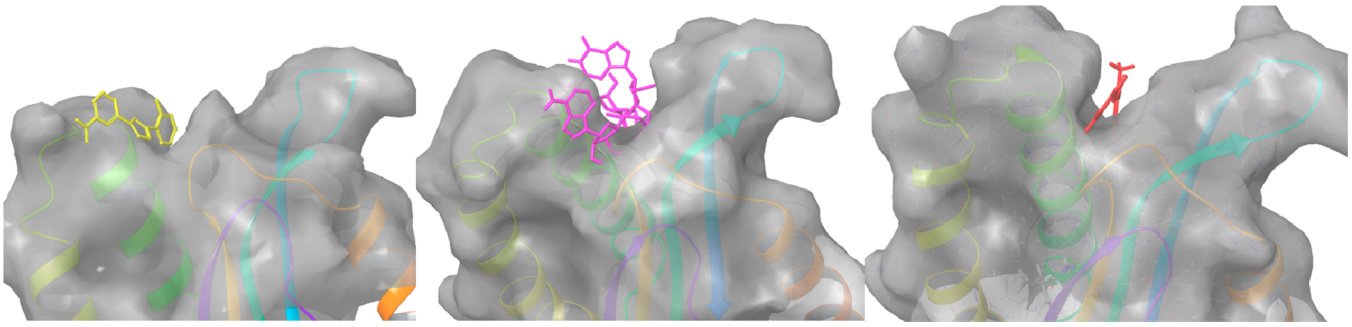

Figure 3. Binding pose of Ataluren (yellow), UGA PTC (purple), and NV2445 (red) into the eRF1 (PDB ID: 2LLX).

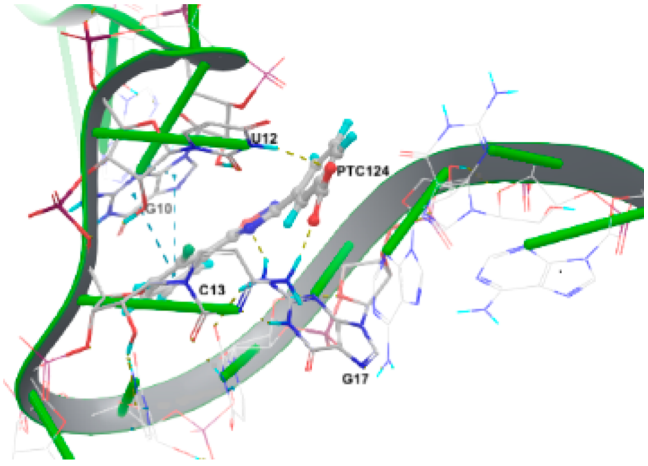

Figure 4. PTC124−mRNA complex generated with IFD. The interactions of $\pi-\pi$ stacking are represented as blue dotted lines; the hydrogen bonds as yellow dotted lines.

one of the 12 "virtually mutated" mRNAs obtained was subjected to IFD and MM-GBSA analysis. The results in terms of docking scores and binding energies are shown in Table 1 together with values referred to the fragment of unchanged CFTR^{opal}-mRNA containing the UGA PTC.

Interestingly, all the replacements were causing a decrease in the value of $\Delta G_{binding}$ negatively affecting the Ataluren/UGA interaction. However, such decrease was ranging between 1.6 and 8.0 kcal/mol when replacement concerned G19, U12, or C13, while it was more prominent, in the range 11.0−15.3 kcal/mol, when the virtual mutation replaced G10 of the mRNA fragment. This suggested an ancillary role of guanine at position −6 with respect to the stop codon, to stabilize the Ataluren−mRNA complex. On the basis of this computational result, we re-examined the mRNA sequences surrounding the premature stop codon for reported experimental studies on the readthrough activity of Ataluren and we found a connection between the successful cases of experimentally proven readthrough and the presence of a guanine at position −6, whereas in unsuccessful or debated cases such ancillary G(−6) was absent.[22]

In summary, modern therapeutic approaches for genetic diseases caused by nonsense mutations aim at bypassing the PTC to allow the synthesis of the full-length functional protein. This requires the use of molecules, such as Ataluren (PTC124), with a readthrough activity as selective as possible. However, although Ataluren's ability to restore the synthesis of the functional protein in the presence of a PTC has been known for some years, there is no certainty on how Ataluren works. With this study we tried to shed light on its probable MOA. Computational studies were performed using bacterial 16S rRNA and the 18S rRNA present at the level of the eukaryotic ribosomal decoding site (site A) and using some aminoglycoside antibiotics and analogue NV2445 as interesting readthrough promoter in order to perform a comparison. Both docking scores and binding energies were significantly higher for aminoglycosides with respect to Ataluren or its analogues. On these bases it is not likely that Ataluren shares the same biological target (i.e., rRNA) with aminoglycosides.

Additionally, the release factor eRF1 involved in protein synthesis termination was examined as an alternative target for Ataluren. The binding energies for the Ataluren/eRF1 and NV2445/eRF1 complexes were three times lower than the affinity between UGA/eRF1. It is therefore difficult to hypothesize that the readthrough of the PTC induced by Ataluren could arise from a direct competition with the eRF1. The low affinity toward these targets agrees with Ataluren's lack of antibacterial activity and the absence of side effects which would arise in the case of interactions with targets involved in regular protein synthesis.

On the other hand, since it is established that binding to RNA fragments can reliably model the binding to actual RNA,[23,24] we performed a docking analysis using a fragment of the model CFTR^{opal}−mRNA containing the UGA PTC, obtaining better results in terms of Ataluren's binding affinity toward the PTC. The $\pi-\pi$ stacking interaction outcomes are fundamental for the achievement of stable complexes. IFD, by means of which the flexibility of the receptor was also considered, highlighted the formation of hydrogen bonds, involved in the further stabilization of the Ataluren−mRNA complex. These results confirmed the likelihood of mRNA as target for Ataluren and are in agreement with the MOA proposed by Jacobson and co-workers,[8] where the insertion of a small molecule (Ataluren or its analogues) could primarily lead to mRNA:tRNA mispairing at codon position 3, where multiple nonstandard mispairings such as A-C, G-G, and A-G are tolerated and provoke the insertion of a near-cognate tRNA.

Moreover, the computational mutagenesis investigation highlighted the importance of guanine present at position −6 with respect to the PTC to stabilize the interaction with the receptor. Indeed, in the absence of this ancillary nucleotide, the estimated binding energies were reduced to less than half with respect to interaction with unchanged CFTR^{opal}−mRNA fragment. These results, combined with the records of the occurrence (or lack) of ancillary G(−6) in the mRNA sequence in successful (or unsuccessful) experimental readthrough, strongly suggest that evaluations for diseases caused

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by nonsense mutation should also assess the genetic context surrounding the PTC, in order to better define the target population for a TRIDs-based therapy.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00558.

Materials and methods (S2−S4), structures of Ataluren's analogues (S7), and tables of results values (S5−S8) (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

MOA, mechanism of action; IFD, induced fit docking; QPLD, quantum polarized ligand docking; eRF1, eukaryotic Release Factor 1; PTC, premature termination codon; MD, molecular dynamics

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