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RESEARCH ARTICLE

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Novel variants of engineered water soluble mu opioid receptors with extensive mutations and removal of cysteines

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Abstract

We have shown that water-soluble variants of the human mu opioid receptor (wsMOR) containing a reduced number of hydrophobic residues at the lipid-facing residues of the transmembrane (TM) helices can be expressed in *E. coli*. In this study, we tested the consequences of increasing the number of mutations on the surface of the transmembrane domain on the receptor's aqueous solubility and ligand binding properties, along with mutation of 11 cysteine residues regardless of their solvent exposure value and location in the protein. We computationally engineered 10 different variants of MOR, and tested four of them for expression in *E. coli*. We found that all four variants were successfully expressed and could be purified in high quantities. The variants have alpha helical structural content similar to that of the native MOR, and they also display binding affinities for the MOR antagonist (naltrexone) similar to the wsMOR variants we engineered previously that contained many fewer mutations. Furthermore, for these full-length variants, the helical content remains unchanged over a wide range of pH values (pH 6 ~ 9). This study demonstrates the flexibility and robustness of the water-soluble MOR variants with respect to additional designed mutations in the TM domain and changes in pH, whereupon the protein's structural integrity and its ligand binding affinity are maintained. These variants of the full-length MOR with less hydrophobic surface residues and less cysteines can be obtained in large amounts from expression in *E. coli* and can serve as novel tools to investigate structure-function relationships of the receptor.

KEYWORDS
computational protein design, G coupled protein receptor, human μ opioid receptor, opioid, water-soluble variant

1 | INTRODUCTION

The high-resolution structures of the opioid receptors have been determined; however, these structures include only the transmembrane domain.[1-5] For the mu opioid receptor, although high-resolution structures of the murine receptor are known, the structure of the human mu opioid receptor (MOR) remains unsolved. Based on the high homology between the human and the mouse MOR, it is possible to leverage structural information from the mouse MOR to generate reliable structures of the human counterpart.[6,7] Such structural models have allowed us to precisely identify residues that are solvent exposed in the transmembrane (TM) domain of the human MOR.[6,7] Replacement of some

Abbreviations: CD, circular dichroism; FL, full-length; KcsA, a prokaryotic potassium channel; MOR, mu opioid receptor; TM, transmembrane; wsMOR, water-soluble variants of the human MOR.

of the hydrophobic amino acid residues enabled us to produce water-soluble analogs of the TM domain (wsMOR-TM) and of the full-length (wsMOR-FL) human MOR in large quantities in *E. coli*.[6-8] Our approach of replacing some of the exterior hydrophobic residues with hydrophilic ones by computational protein design has allowed us to produce large quantities of wsMOR in *E. coli*. This approach greatly facilitates the study of the interaction of the receptor with various opioid ligands. We are able to investigate such interactions in aqueous media without membranes or micelles, and we can study these interactions with novel protein functionalized electronic sensor systems fabricated with graphene-enabled technologies.[6,9,10] We recently added the N and C terminal domains to the wsMOR variant, which had been designed using only the TM domain. The resulting full length construct, wsMOR-FL, retains important properties, including receptor expression in *E. coli* and binding affinity for opioid ligands.[8]

It is well accepted that the hydrophobic residues at the surface of the TM domain, particularly those located in the lipid-facing positions, contribute to the functional and structural integrity of this membrane protein by providing favorable interactions with the lipid bilayer. How the number and identities of these hydrophobic residues affect the binding capabilities of particular receptors has been less studied. In this study, we tested the consequences of designed mutations on the surface of the water-soluble variants, with particular attention to the expression in *E. coli* and to the receptor's ligand binding affinity.

2 | MATERIAL AND METHODS

UniProtKB server (P35372) was used to obtain the human MOR sequence.[11] N-terminal modified MORs in HEK293 cells, fluorescent naltrexone and were obtained from Cisbio (Bedford, MA). All other compounds or reagents were analytical grade and obtained either from Sigma-Aldrich (St. Louis, MO) or from the central supply facility at the University of Pennsylvania.

2.1 | wsMOR variant engineering and expression

The receptor variants designed in this study include both native N and C termini. A computational formalism was applied to identify the amino acid probabilities from a given structure according to our previously described entropy-based formalism for the transmembrane portion of human MOR (288 residues, comprising amino acids 66-353).[6,7] In contrast to our previous design, additional hydrophobic exposed residues were selected to be part of the design protocol. The final number of TM positions open for mutations were 100 out of 288 (~34.7%). This percentage is comparable to previous efforts related to the design of a water-soluble variant of the bacterial KcsA, a prokaryotic potassium channel where ~28% of the positions were mutated,[12] but greater than our previous study of wsMORs in which the percentage of open positions was only ~18.5%. (6) We retained the key residues forming part of the ligand-binding site of MOR[7,11]: K235$^{5.40}$, Y150$^{3.33}$, H299$^{6.52}$, V302$^{6.55}$, I298$^{6.51}$, M153$^{3.36}$, Y328$^{7.42}$, W295$^{6.48}$, and D149$^{3.32}$ (superscripts indicate the Ballesteros-Weinstein index use for the positions in the TM domain; Nter, ICL2, and ECL2 are used to denote positions located at the N-terminus, intracellular loop 2, and extracellular loop 2, respectively). In addition, 11 cysteine residues present in the protein were selected for designed mutation regardless of their solvent exposure value and location in the protein, so as to avoid issues with un-expected cysteine oxidation and disulfide bond formation. We retained residues C142$^{3.25}$ and C219^{ECL2}, which connect the third transmembrane helix (TM3) and the second extracellular loop (ECL2) via a conserved disulfide bridge. Proline residues were excluded from the design calculation since they are often structurally important in transmembrane proteins. During the computational design, 10 different structural templates were utilized, model A to J (Figure 1). For each template, a new sequence was obtained using the protocol described previously, based on a sequence entropy-based formalism. The results were 10 newly designed sequences of the TM domains that were further evaluated using a modeling process to ensure a robust similarity in structure.

In order to select one sequence from the 10 designed wsMORs, secondary structure prediction was performed using PORTER, a well-known server for the prediction of the protein secondary structure content.[13] No difference of the predicted secondary structure of the different sequences was found. Next we calculated formation of salt-bridges based on the following criteria: the distance between any of the oxygen atoms of the acidic residues, DE, and any of the nitrogen atoms of the basic residues, KRH, should be less than 3.2 Å. Based on the predicted numbers of salt-bridges formed and the numbers of ionizable residues not forming a salt-bridge, we found that the wsMOR-FL_G has most favorable salt bridges followed by wsMOR-FL_D, wsMOR-FL_H, and wsMOR-FL_I, therefore, these four variants were selected for expression and purification and for binding assays. The sequence with the most favorable expression yield and better overall binding affinity was subsequently selected for further characterization and comparison of the full-length and transmembrane (FL) and the only-transmembrane domain (TM). These receptors were over-expressed with N-terminal His-tags in *E. coli* BL21(DE3) cells (EMD/Novagen) and then purified using a nickel purification column as we reported previously.[6]

2.2 | Naltrexone binding

The affinities of the wsMOR-FLs with naltrexone, an antagonist of MOR, was determined as previously reported.[6-8] This methodology uses a homogeneous time-resolved fluorescence based binding assay with fluorescent labeled naltrexone and Tag-lite mu opioid cells (Cisbio Bioassays, Bedford, MA) for binding competition purposes to determine the binding affinity. Affinities were estimated by obtaining a concentration-response association using the Cheng-Prusoff equation by GraphPad 8.2.1 (GraphPad Software, San Diego, CA). Three separate repeats were performed to ensure reproducibility.

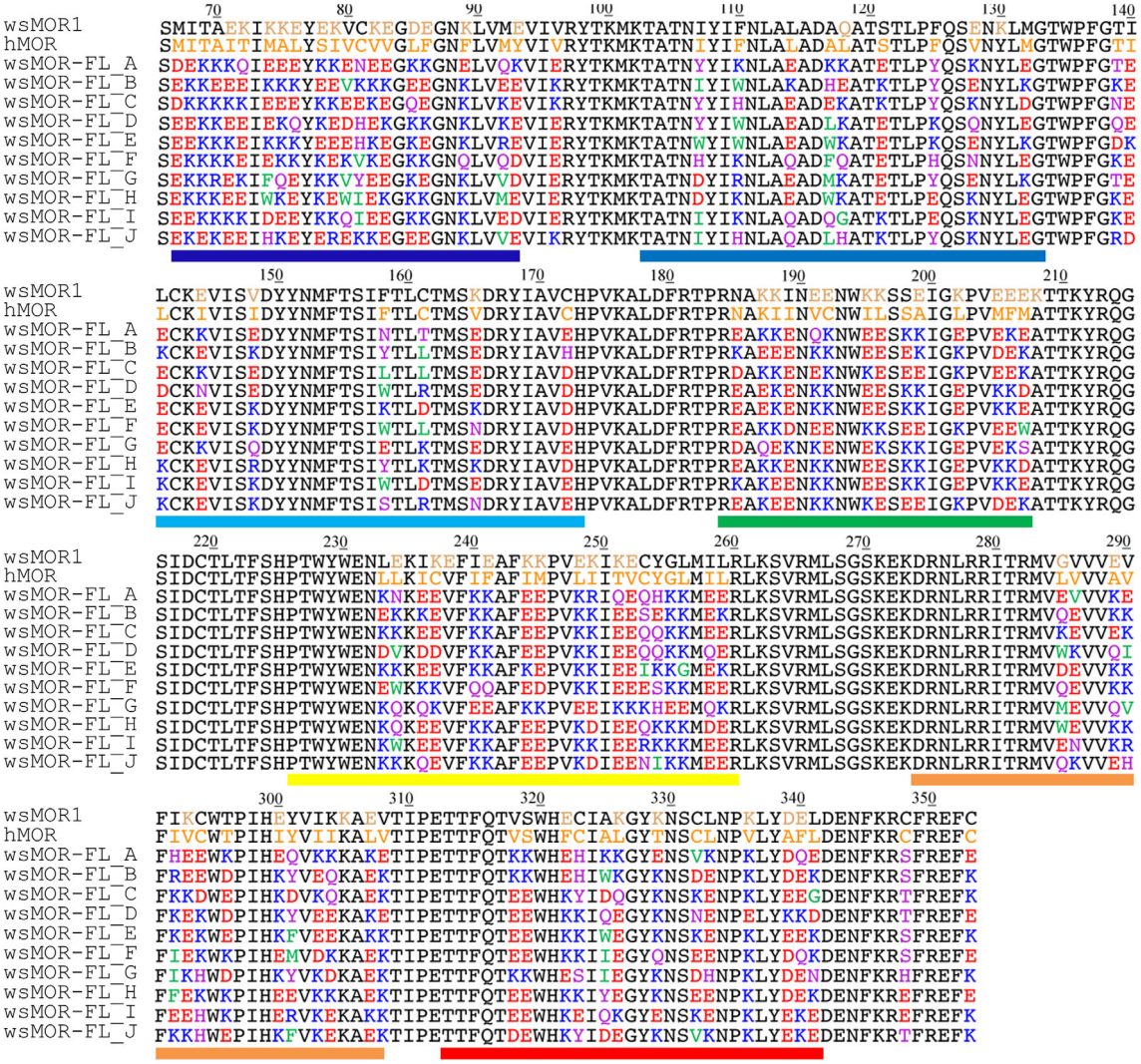

FIGURE 1 The transmembrane domains of the sequences of the 10 computationally engineered water-soluble variants of the human mu opioid receptors (wsMORs) aligned together with the native MOR and one of our initially designed wsMOR variants (wsMOR1) to indicate residues being mutated. The 100 new positions in the transmembrane domain targeted for the computational design are colored orange in the hMOR sequence, which includes all the positions bearing a cysteine residue except for those forming the highly conserved disulfide bond between TM3 and ECL2 ($C142^{3.25}$ and $C219^{ECL2}$, respectively). The 10 new sequences are named as wsMOR-FL_A ~ J, where acidic residues are colored red, basic residues are colored blue, hydrophobic residues are colored green, and hydrophilic residues are colored magenta. The different transmembrane helices are indicated in Figure 2. TM, transmembrane domain; FL, full length; MOR, mu opioid receptor; hMOR, native human mu opioid receptor; ECL, extracellular loop; wsMOR, water soluble mu opioid receptor

2.3 | Structure and stability

Circular dichroism (CD) (Chirascan, Applied Photophysics Limited, Leatherhead, UK) was used to determine the secondary structure of wsMOR-FL-G and wsMOR-TM-G with a scanning speed of 1 nm/s (1 mm path length) as we described previously.[6,7] Signals from the buffer without wsMOR variants were subtracted as a correction. Each experiment was repeated at least three times to ensure reproducibility. CD spectra for wsMOR-FL-G and wsMOR-TM-G at various pHs and temperatures were measured with 6 μM of wsMOR-FL-G in buffer containing 5 mM sodium phosphate (pH 7.0), from 10°C to 90°C. GraphPad Prism (version 8.2.1 GraphPad Software, Inc. La Jolla) was used for data analysis and graph generating purposes.

3 | RESULTS

3.1 | wsMOR-FL, engineering, expression, and purification

The sequences of the engineered variants of wsMOR are presented in Figure 1 with comparison to one of the previously engineered variants.[6,7] The structure similarities of the engineered wsMORs are presented in Figure 2. The details of the replacement residues in each cysteine position are presented in Table 1 and their relative positions are presented in Figure 2. As indicated in Figure 3A, all the selected sequences were expressed in an *E. coli* expression system. The yield of the receptor was ~20 mg/L of flask culture. All of them could be

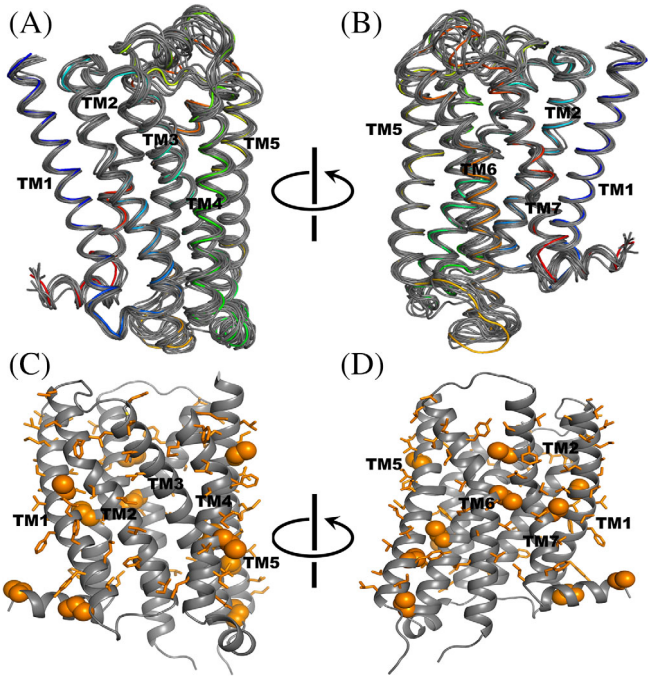

FIGURE 2 Lateral views of the 10 structural models of TM domain of MOR utilized during the computational protein design. Panels A and B are the rotated images of the 10 models. One structure is colored in a rainbow fashion (from blue to red) going from the N-terminus to the C-terminus; the different TM helices are also indicated (TM1 to TM7). Due to the backbone dependency of the computational design protocol, each structure generated a different set of probabilities at each position, which is reflected in the final designed sequences presented in Figure 1, namely wsMOR-FL_A ~ J. Panels C and D are the rotated images of the native MOR structure (4DKL.pdb). The positions targeted during the computational design are indicated as orange sticks; positions where those bearing a cysteine residue are depicted as spheres. TM, transmembrane domain; MOR, mu opioid receptor; wsMOR, water soluble mu opioid receptor

purified for binding assay purposes with a recovery rate of more than 50% in a milligram scale ranging from 4.5 to 7 mg/mL in a buffer condition of 10 mM sodium phosphate and 0.01% SDS. wsMOR-FL_G demonstrated much higher yields as compared to the rest of three variants. The purified wsMOR-FL_G in buffer solution demonstrated good solubility without visible aggregation, and only one clear visible band was identified in the SDS-PAGE gel (Figure 3B), suggesting high purity.

3.2 | Binding assay for the variants of wsMOR-FLs

The binding assay using naltrexone, as a competitive ligand reported previously, indicated the dissociation constants of naltrexone were 82 nM for sequence wsMOR-FL_D, 89 nM for sequence wsMOR-FL_G, 165 nM for sequence wsMOR-FL_H, and 107 nM for sequence wsMOR-FL_I (Figure 4), which are comparable with those from our previous variants (either the transmembrane domain or the full-length

TABLE 1 Changes of cysteine residues in each variant of wsMOR

	Variants of wsMORs									
	A	B	C	D	E	F	G	H	I	J
C81	N	K	E	H	H	V	Y	I	I	K
C161	T	L	L	R	D	L	K	K	D	R
C172	E	H	D	D	D	D	E	D	E	E
C192	K	K	K	K	K	E	E	K	K	K
C237	E	E	E	D	E	K	K	E	E	E
C253	Q	S	Q	Q	I	E	K	Q	R	N
C294	E	E	D	K	K	K	H	K	H	H
C323	H	H	Y	K	K	K	S	K	E	Y
C332	V	D	K	N	K	E	D	E	K	V
C348	S	S	T	T	S	S	H	E	E	T
C353	E	K	K	E	K	E	K	E	K	K

Note: The positions bearing a cysteine residue in the transmembrane domain of the native human mu opioid receptor (MOR) sequence are indicated. The computationally designed water soluble MOR (wsMOR) for each position in the different 10 sequences (A-J) is indicated.

variant), obtained using the same binding estimation method. However, both sequences (wsMOR-FL_D and wsMOR-FL_G) demonstrated slightly higher affinity with naltrexone.

3.3 | Secondary structure and stability of wsMOR-FL_G and wsMOR-TM_G

Based on the protein yield and binding affinity screening, we found that wsMOR-FL_G displays the highest protein expression yield and higher naltrexone affinity; consequently, the system wsMOR-FL_G was selected for further characterization. The helical secondary structural content was estimated using circular dichroic spectroscopy over the range from 205 to 260 nm. Both wsMOR-TM_G and wsMOR-TM_G demonstrated an alpha-helical protein. Upon changing pH from 6.5 ~ 9.1, there is no significant change in helical content (Figure 5A for wsMOR-TM_G). With change in temperature, the wsMOR-FL_G variant remains relatively stable. The transmembrane portion, wsMOR-TM_G, has a melting temperature around 67°C (Figure 5B).

4 | DISCUSSION

In this study, it is remarkable that despite the extensive mutations introduced in lipid-facing transmembrane positions of MOR by computational design approaches, the binding affinity capabilities of the receptor remain relatively unchanged. It is very likely that the careful selection of the mutated sites may contribute to the retention of the binding capabilities of the variant investigated here. In addition, mutation of the cysteine residues did not significantly affect the ligand binding affinity. This study further confirms our previous findings that the receptor expression is not affected by the inclusion of the native

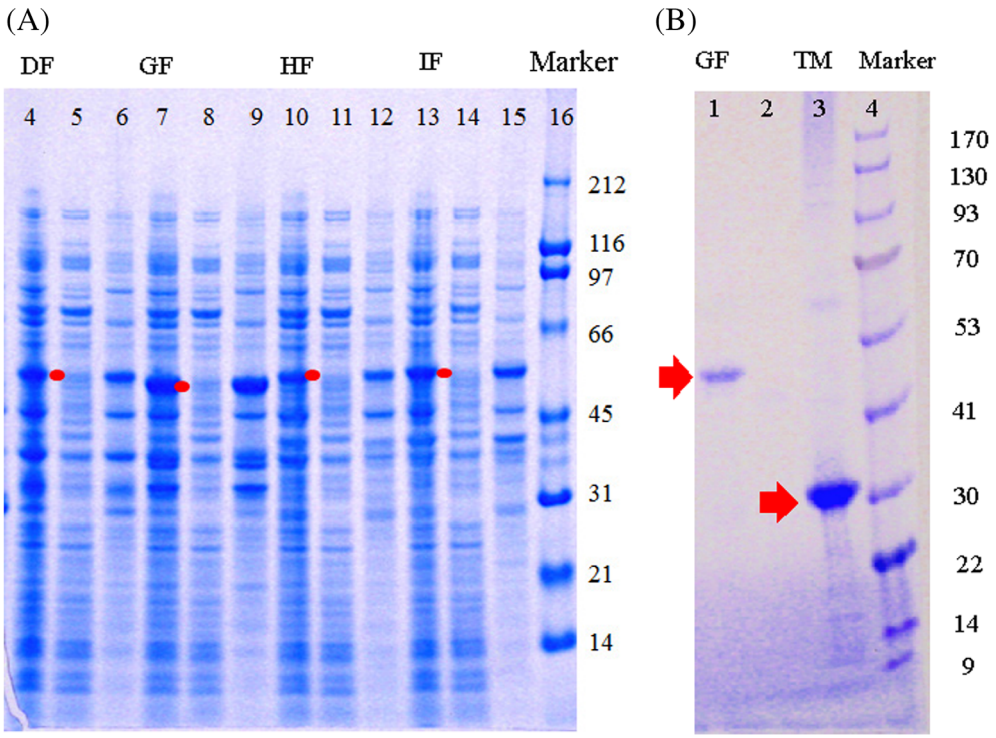

FIGURE 3 SDS-PAGE gel of the water-soluble variants of human MOR, wsMOR-FL_D, wsMOR-FL_G, wsMOR-FL_H, and wsMOR-FL_I and examples of the purified variants, wsMOR-FL_G, and the transmembrane domain. Red dots in the left panel (image A) indicate the desired protein bands. These receptors were subsequently purified for affinity determination and for further biophysical characterization. Lanes 4 to 6 are for the sequence of wsMOR-FL_D (DF, from left to right, total cell lysate, soluble proteins, insoluble proteins). Lanes 7 to 9 correspond to the sequence of wsMOR-FL_G (GF, total, soluble, insoluble). Lanes 10 to 12 correspond to the wsMOR-FL_H system (HF, total, soluble, insoluble). Lanes 13 to 15 are for the sequence of wsMOR-FL_I (IF, total, soluble, insoluble). Lane 16 shows molecular weight markers. Red arrows in image B indicates examples of the purified wsMOR-FL_G (lane 1) and a transmembrane domain of wsMOR (lane 3). Lane 2 is a blank control and lane 4 shows makers. FL, full length; MOR, mu opioid receptor; wsMOR, water soluble mu opioid receptor

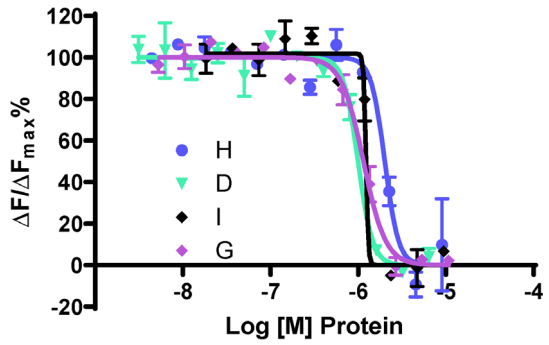

FIGURE 4 Competition binding assay between native human MOR expressed in HEK293 cells and the newly designed water -soluble variants, wsMOR-FL_H, (H), wsMOR-FL_D (D), wsMOR-FL_I (I), and wsMOR-FL_G (G). Data is used to calculate fluorescence emission ratios, and represent the mean ± SE of the mean of quadruplicates. HTRF signal ratio was calculated as a two-wavelength signal ratio: [intensity (665 nm)/intensity (620 nm)]. ΔF is used for the comparison of different runs of the same assay which reflects the signal to background of the assay. $\Delta F = [(\text{Ratio}_{\text{sample}} - \text{Ratio}_{\text{backgroud}})/\text{Ratio}_{\text{backgroud}}](\%)$. ΔF_{MAX} is the largest ratio for the background. MOR, mu opioid receptor; wsMOR, water soluble mu opioid receptor; FL, full length; HEK, human embryonic kidney; HTRF, homogeneous time resolved fluorescence

N and C terminal domains, thus we are able to obtain additional water-soluble variants of full-length human MOR in large quantities.

4.1 | Over-expression of full-length variants of human MOR

We recently reported that engineered full-length human MOR can be over-expressed in *E. coli*, and that its overall secondary structure and ligand binding affinity are similar to that of the native human MOR.[8] The addition of native N and C terminal domains to our previously engineered TM domains of wsMORs did not affect expression or purification; and this study corroborates those findings. The newly designed variants had numerous additional mutations on the surface of the transmembrane domain of the receptor, including replacement of a number of the cysteine residues. These receptor variants offer additional tools to study structure-function relationships of the human MOR in a lipid free system. The structure, function, and the interplay with the transmembrane domain of the N and C terminal domains are not clear yet. They may play critical roles in opioid ligand recognition and signal transduction by interaction with the G-protein and

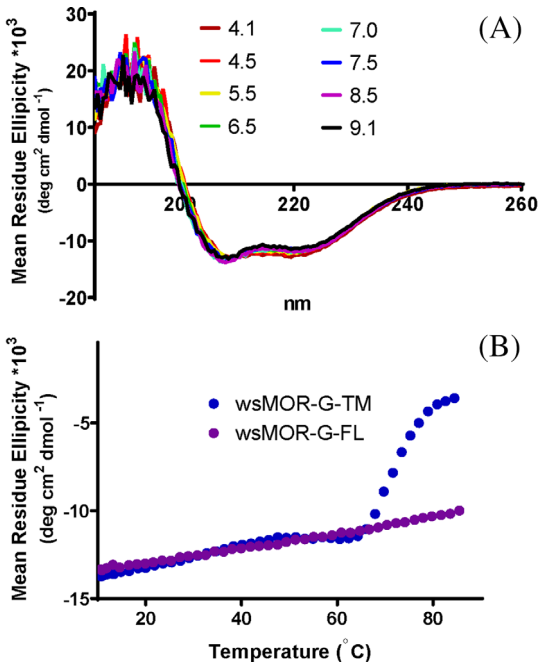

FIGURE 5 A, Circular dichroism (CD) spectra for the wsMOR-FL_G variant. No significant CD spectra changes are seen within the pH range from 6.5 ~ 9, indicating a stable secondary structure within the pH tested. However, we observed some secondary structural features begin to change when the pH is lower than 6.5. B, Mean residue ellipticity at 222 nm of wsMOR-FL_G. (the buffer composition is already described in Methods and Materials) as a function of temperature, from 10 to 90°C. The spectrum of wsMOR-TM_G showed significant change near 62°C and an almost complete loss in molar ellipticity at 90°C. The loss of the molar ellipticity is less severe in the case of the wsMOR-FL_G. TM, transmembrane domain; FL, full length; MOR, mu opioid receptor; wsMOR, water soluble mu opioid receptor

beta-arrestin recruitment.[14,15] These new receptor variants could potentially help to investigate and dissect the function of the N and C terminal residues, and how they contribute to the overall protein stability and the tertiary structure and function of the MOR.

4.2 | The structure and thermostability of the wsMOR-FL_G

wsMOR-FL_G showed a predominantly helical structure, which is comparable with that of variants that we reported on previously.[6-8] This result indicates that extra mutations on the surface of the protein and replacement of some of the cysteines did not disrupt the basic tertiary structure of the receptor. The predictions from the computational design of the protein suggested the possibility of human MOR to tolerate a larger number of mutations, which we have now demonstrated experimentally. These findings are consistent with our recent report that the thermostability of the wsMORs was improved by the presence of the N and C terminal domains. Changing the residues on the surface of the protein can change the thermostability of the receptor, however, adding sucrose could improve the thermostability significantly as we have demonstrated in the past.[7] Interestingly, the wsMOR-FL_G variant remained stable over a wide range of pH values without changing its secondary structural content. However, in an acid environment (pH less than 6.5), such stability was slightly disrupted. The implication of this effect may be relevant for the in vivo function of the receptor when exposed to acidic environments. These data are guiding us in performing further computational evaluations of the engineered variants in solution conditions using molecular dynamic simulations. This is an ongoing project which we will report on soon.

4.3 | Opioid binding capability

The results clearly indicate that all four of the new wsMOR-FLs have comparable ligand binding affinity (nM range) as the variants engineered previously (which all have similar ligand binding affinities around 70 nM).[7] Using the various biosensors developed by our group which include graphene based biosensors and surface plasmon resonance based biosensors, the determined affinities for these designed variants of wsMORs are close to the affinities for the native MOR in the lipid environment.[10,16,17] The different results in the affinity determinations are caused by differences in sensitivity, since the biosensors have high sensitivity and a high signal to noise ratio. The key point here is that the opioid binding capacity is well-preserved, despite a massive mutation strategy for the residues on the surface of the protein, as long as the opioid binding pocket remains intact. These engineered water soluble MOR variants provide useful tools to further investigate molecular pharmacological signaling pathways in the absence of lipid. One example is the interaction of the receptor with guanine nucleotide-binding proteins, which is ongoing in our research group.

4.4 | The implications and applications

As compared to our recently published work on the full-length version of the wsMOR,[8] there are multiple scientific implication and applications for this new effort in addition to these we have discussed or demonstrated in the past.[6,7,9,16,17] First of all, in this is proof-of-concept, additional mutations on the surface of lipid-facing hydrophobic positions to resemble more the sequence identity of globular protein of the same size, were introduced. We wanted to know if the increase of mutated position could maintain the receptor's overall structure and ligand binding capability. In addition to the usefulness of the variants of water soluble protein that has been discussed by us and others,[9,10,16-18] the availability of minimal cysteine variants offers several opportunities for the study of the receptor's structural and functional relationships. Recent studies have used similar approaches to study the conformational dynamics of the β_2AR using NMR and single-molecule fluorescence spectroscopy.[19-22] One of the possibilities is to introduce a cysteine at a specific location of the engineered

minimal cysteine wsMOR to report ligand binding affinity and the conformational dynamics in real-time. We are currently working on one variant based on wsMOR-TM-G to explore such possibility.

4.5 | The limitation of the study

The major limitation of this study is that we provide only limited information on the tertiary structure of the variants of wsMOR. It is possible that such massive mutations and removal of cysteine residues could potentially have caused subtle structure changes that would have been too small to be detected by the technologies used in this study. Further characterization of the variants presented in this study is warranted. More studies are also needed to determine whether the subtle structural changes that occur under low pH are caused by introducing more polar residues on the surface of the receptor. It is important to note that some mutations in the receptor related to some disease states and changes opioid ligand affinity and/or affinity. For example, point mutations of S196A$^{4.45}$ in murine mu opioid receptor could change the opioid antagonist properties.[23] Mutation of residue in the binding pocket will change the receptor's affinity or interactions with opioid ligand, one of the example is N152$^{3.35}$ (N150$^{3.35}$ in the mouse MOR) as we demonstrated in the past.[24] N152A$^{3.35}$ (N150A$^{3.35}$ in the mouse MOR) point mutation can change fentanyl affinity significantly.[25,26] Mutations in residues such as D116$^{2.50}$, D149$^{3.32}$ and H299$^{6.52}$, alter the ligand binding capacities of the receptor toward different ligands.[27,28] Also, some mutations, including S198$^{4.45}$, have been suggested to modify the receptors' function by changing its ability to form dimers. Additionally, single point mutations have been found to modulate the activity of the receptor, including D166$^{3.49}$ and N152$^{3.35}$. Furthermore, naturally occurring mutations have been shown to modulate different aspects of the receptor's functions, including S42T^{Nter}, L85I$^{1.47}$, R181C^{ICL2}, C192F$^{4.48}$.[29] Lastly, position N152$^{3.35}$ is critical to modulate the biased agonism of MOR, that is, the N152A$^{3.35}$ mutation renders MOR as a β-arrestin-biased receptor.[30] Figure 6 presents these key residues for easy understanding purposes. Though this is not the focus of this study, the designed variant could potentially be used to investigate whether such specific mutation will change the affinities or potencies of endorphin in the high resolution molecular level. In our receptor engineering, we avoided mutating any residues in the binding pocket based on the best available structural information. The role of the lipid membrane in providing the physiological milieu for the receptor proper functions has been also extensively investigated.[31,32] Due to the water-soluble character of the variant investigated here, the lack of any lipid membrane eliminates the possible role that the lipids play in mediating the different functions of the receptor, including ligand binding (either orthosteric or allosteric) and dimer formation.[31,33]

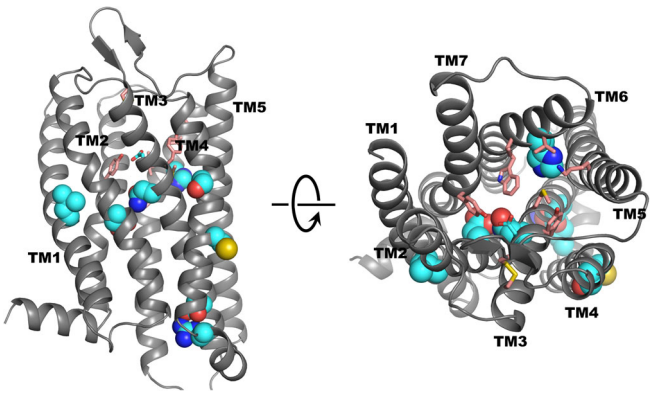

FIGURE 6 The position in salmon and sticks are the residues forming part of the ligand-binding site of MOR that were retained: K235$^{5.40}$, Y150$^{3.33}$, H299$^{6.52}$, V302$^{6.55}$, I298$^{6.51}$, M153$^{3.36}$, Y328$^{7.42}$, W295$^{6.48}$, and D149$^{3.32}$. Residues in cyan and spheres are receptor loci known to modulate the function of MOR when mutated: D116$^{2.50}$, D149$^{3.32}$, H299$^{6.52}$, D166$^{3.49}$, N152$^{3.35}$, S196$^{4.45}$. Also naturally occurring single point mutations are indicated in cyan: L85I$^{1.47}$, R181C^{ICL2}, C192F$^{4.48}$. The structure corresponds to the murine MOR with the protein data bank accession code 4DKL, however, the residue numbers correspond to the human MOR. These are the rotated images for a better presentation purpose

5 | CONCLUSION

In conclusion, the binding capability of the engineered wsMORs was not significantly affected by replacing additional hydrophobic residues with hydrophilic residues on the surface of the transmembrane domain. Binding capability was also not significantly affected by replacing a number of the cysteine residues in the receptor determined by the same methodologies. This indicates that these residues are not essential for opioid recognition and binding. These newly engineered full-length variants of wsMOR offer novel tools to further investigate the molecular pharmacological signaling pathways of MOR in the absence of lipid. These include such things as the interaction of the receptor with guanine nucleotide-binding proteins.

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PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/prot.26160.

DATA AVAILABILITY STATEMENT

Original data is available for a reasonable request to the corresponding authors.

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