Molecular Modeling Workflow for Identification of Advanced Glycation End Products as Ligands for Bitter Taste Receptors

Ryan Howard

BSc (Dent) Report

ABSTRACT

Bitter taste in humans is mediated by sub-family of G-Protein Coupled Receptors (GPCRs) consisting of 25 receptors called bitter taste receptors (T2Rs), that are expressed mainly in taste buds, but have been found to be expressed extraorally as well. Extraoral T2Rs have been suggested to play a role in a variety of pathophysiologic conditions such as in upper and lower airway diseases. Advanced glycation end products (AGEs) comprise a highly variable group of molecules that can be formed by either exogenous or endogenous means, through the reaction of reducing sugars such as glucose with amino groups in the sidechains of amino acids of proteins for example. AGEs have been extensively investigated for their possible contributory role in a variety of pathologic conditions such as cardiovascular disease, diabetes mellitus, and chronic kidney disease. A significant number of agonists for bitter taste receptors have been reported, but very few characterized antagonists are known. Structure-function studies of T2Rs and their ligands is complicated by the fact that, like the vast majority of GPCRs, no high-resolution structure of any of the 25 T2Rs has been solved to date. We herein report the use of a molecular modeling workflow whereby a bitter taste receptor model and a library of virtual ligands are generated, and the binding potential of the ligand library with the T2R model is rapidly screened. The use of molecular scoring functions allows the ligands to be ranked and predicted interactions examined on a molecular level. Top candidates were then characterized further by *in vitro* calcium assays to determine the presence of any functional T2R interaction capability. Glyoxal-derived lysine dimer (GOLD) is identified as a candidate for future study.

INTRODUCTION

Taste is one of the five basic senses that are used to describe the perceptual abilities of humans, along with sight, hearing, smell, and touch.[1] Taste sensation thereby plays a critical role in the nervous system's responsibility of providing trustworthy information for interpretation regarding the external environment.[1] The sensation of taste is further broken down into five major classifications by the characteristic of tastes sensed, namely sweet, salty, bitter, sour, and umami.[1, 2] Each of these basic taste modalities plays a unique role in the body's discernment as to the attributes of substances taken into the oral cavity. For example, while humans exhibit an innate preference for sweet tasting substances[2] and recognize them as being highly caloric,[1] it has been shown that exposure to bitter tasting substances causes the development of a bitter rejection response consisting of aversive reflexes, purportedly to prevent the digestion of toxic or poisonous materials.[1-3]

Although gustation or taste is one of the five basic senses for mammals, the biological mechanisms that allow its perception have been historically somewhat less developed relative to the other senses.[2] The perception of taste is facilitated by taste receptor cells, which are aggregated together in the form of taste buds.[2] Taste buds can be found throughout the oral cavity, and are plentiful on the dorsal surface of the tongue as constituents of one of three types of papilla: foliate, fungiform, and circumvallate papillae.[2, 4] The taste receptor cells that comprise a taste bud express receptors that allow the recognition of water soluble ligands or tastants to induce a signaling cascade that

relays information to the central nervous system regarding the quality of the material in the oral cavity.[4]

Bitter taste in humans is mediated by a sub-family of G Protein-Coupled Receptors (GPCRs) consisting of 25 receptors called bitter taste receptors (T2Rs) that are expressed by certain subsets of taste receptor cells present in the oral cavity.[4, 5] While T2Rs are principally responsible for recognizing bitter tastants in the oral cavity with exquisite specificity, they have also been found to be expressed in a variety of extraoral tissues, including respiratory and gastrointestinal tissues.[6-9] Although some studies have suggested pathophysiologic roles for T2Rs in extraoral tissues in a variety of upper and lower airway diseases and others, the role of these receptors beyond the oral cavity remains a largely untapped area of research.[9]

Advanced glycation end products (AGEs) represent a broad range of molecules formed either exogenously or endogenously by the non-enzymatic reaction of reducing sugars such as glucose with free amino groups from side-chains of proteins and other macromolecules.[10, 11] The reaction between reducing sugars and amino groups that forms AGEs is called the Maillard or Browning reaction, and it involves transformation through a process of Schiff's base formation and subsequently the formation of a stable Amadori product, followed by finally the formation of the AGE.[10, 11] It should be noted that while this process can take weeks endogenously, exogenous formation of AGE's in foods is facilitated much more readily by cooking them under dry conditions at high temperatures for extended periods of time.[10]

The role of AGEs in human pathophysiology has been studied extensively, and they have been implicated to a certain extent in a variety of conditions such as chronic

kidney disease, cardiovascular disease, several neurodegenerative diseases, and diabetes mellitus.[10] While the specific roles of AGE's in these conditions and others remain under investigation, several broad mechanisms for the pathophysiology of AGEs have been suggested, including: (i) the formation of crosslinks in long-lived proteins such as collagen, leading to compromises in protein functionality and normal degradation processes that are subsequently implicated in tissue stiffness and organ dysfunction[10, 11] and (ii) interaction with specific cell surface receptors such as Receptor for advanced glycation end products (RAGE) to initiate intracellular pathways that induce an increase in production of reactive oxygen species (ROS) and inflammatory cytokines.[10, 11]

Bitter taste receptors (T2Rs) have been the subject of significant study for nearly two decades, with research intensifying more recently as the expression of these receptors in extraoral tissues became apparent.[6-8] However, as is typical with GPCRs and other membrane proteins, progress on elucidating functions based on structural studies has been impeded with the lack of a high resolution 3D structure of a T2R.[12] Recently, a number of high-resolution structures of GPCRs have been solved, a breakthrough that was allowed by several technical advances such as high concentration expression, flexible domain stabilization, lipid-based crystallography, and the use of micro-diffraction X-ray sources.[13-16] Access to high-resolution GPCR structures such as β_2-Adrenergic Receptor (β_2AR) and Rhodopsin has in turn opened the door for the structures of other related receptors in this family to be estimated with some success using the computer driven process of homology modeling.[17] Furthermore, the interaction of these protein models with libraries of small-molecule ligand candidates can be

simulated as well, giving valuable insight and guidance in directing subsequent *in vitro* studies.

We herein report the use of a homology modeling and virtual ligand docking workflow (Figure 1) to drive the screening of a chemical library of AGEs for their interaction ability with a T2R4 model, followed by the use of *in vitro* calcium mobilization assays to assess the potential agonistic or antagonistic activity of high-ranking hits from our virtual screen.

ABBREVIATIONS:

AGEs, Advanced glycation end products; GPCR, G protein-coupled receptor; RAGE, Receptor for advanced glycation end products; ROS, reactive oxygen species; T2Rs, Bitter taste receptors; β_2AR, β_2-Adrenergic Receptor; GOLD, Glyoxal-derived lysine dimer; CML, Nε-Carboxymethyl-lysine; bis-CML, Nα,Nα-bis(carboxymethyl)-L-lysine.

EXPERIMENTAL PROCEDURES

Materials.

DMEM/F-12, trypsin/EDTA, calcium sensitive dye Fluo-4 NW and probenecid were purchased from Invitrogen. GOLD was purchased from PolyPeptide Group. CML, Quinine, and Trypan Blue Solution, were purchased from Sigma. Common chemicals were purchased from either Sigma or Fisher. All chemicals used were of analytical grade and used without further purification.

Molecular Modeling.

The T2R4 amino acid sequence was obtained from NCBI. All modeling procedures were carried out using BIOVIA Discovery Studio. Two GPCR homologs

were used to generate two models of the T2R4. The first T2R4 model was generated using a crystal structure of an active model of opsin in complex with a C-terminal peptide (Protein Data Bank code 3DQB), and the second was generated using a crystal structure of inactive rhodopsin (Protein Data Bank code 1U19). Modeling began with sequence alignment to the chosen homolog template, which was then used to create a series of 20 preliminary homology models. The preliminary model with the lowest predicted energy was checked for any gaps and necessity for loop optimization in the Discovery Studio Workspace. The model then underwent 2500 iterations of minimization using the Smart Minimizer (1000 steps of Steepest Descent with an RMS tolerance of 3.0 followed by Conjugate Gradient minimization). Quality analysis of the completed model was carried out using Procheck[(18)] available from the SWISS-MODEL Workspace.

Ligand-Receptor Docking.

The model used to obtain the ligand docking results reported herein was constructed from a crystal structure of inactive rhodopsin (Protein Data Bank Code 1U19) using the ITASSER Server and validated *in vitro* via mutational analysis as previously reported.[(19)] The ligand library used for docking consisted of 23 AGEs (Table 1) as well as Quinine as an agonist control and bis-CML as an inverse agonist control given their previously characterized interaction capability with T2R4.[(19)]This library was prepared by compiling chemical structures manually drawn with appropriate formal charges and stereochemistry using the PubChem Sketcher tool or from the PubChem Database, where available. Ligands were minimized prior to docking using the Smart Minimization protocol (1000 steps of Steepest Descent with an RMS tolerance of 3.0, followed by Conjugate Gradient). A binding sphere with a radius of 14.9Å was defined

using the guidance of residues critical for quinine binding as previously reported.[19] Docking of all 25 putative ligands was carried out using the CDOCKER algorithm with 30 random ligand conformations to assume and 30 orientations to refine, with the top ten poses reported. Following initial docking of ligands, all poses were further refined using *in situ* ligand minimization, allowing localized relaxations in the structure of the receptor as well as of the ligand.

Ligand Pose Analysis.

Ligand poses were analyzed using seven scoring functions. These included LigScore1, LigScore 2, PLP1, PLP2, Jain, Ludi Energy Estimate 1 and Ludi Energy Estimate 2. Analysis of ligand poses achieved in docking was carried out by employing a "rank-by-rank" strategy.[20] A score was assigned to each of the top ten saved ligand poses by each of the seven previously mentioned scoring functions, and these scores were then ranked from 1-10 for each ligand. To determine the top scoring pose for each ligand overall, the rankings of each pose by each scoring function were averaged, and the pose with the highest mean ranking was selected for comparison with the highest ranking pose of each of the other docked ligands. The highest scoring pose of each ligand compared to the highest scoring pose of the other ligands using another round of averaged ranking using the same set of scoring functions was used to generate a final ranking of all ligands in the pool (Table 1). A Ludi Energy Estimate 3 score was calculated for each top scoring ligand pose in order to allow the prediction of a K_d value for each, using the formula Ludi Energy Estimate 3 = $-100\log K_d$; these values are also listed in Table 1.[21]

Calcium Mobilization Assays.

After the rank-by-rank scoring method as previously described was used to assess

the simulated potential interaction ability of the full AGE ligand pool, GOLD and CML were chosen as potential T2R4 binding partners to further characterize *in vitro.* This functional characterization was carried out using the Fluo-4 NW calcium assay kit to measure transient changes in intracellular calcium levels as a result of treatment of the cells with various concentrations and combinations of the ligand compounds. Calcium assays were carried out using a HEK293T stably expressing T2R4 and Gα16/44 as previously described, and HEK293T cells as a negative control.[22] After cells reached 90% confluence a viable cell count was taken using a Bio-Rad TC10 automated cell counter, and $1x10^5$ cells/well were plated in a 96-well clear bottom black-walled plate and incubated 16h at 37°C in a CO_2 incubator. Cell media was removed and replaced with 100µL of Fluo-4 NW dye/well, and cells were incubated at 37°C for 40 minutes. Fluo-4 NW dye was prepared as directed by the manufacturer. Lyophilized dye was dissolved in 10mL of assay buffer (1X Hank's Balanced Salt Solution, 20 mM HEPES) and probenecid was added at a concentration of 2.5 mM to prevent leakage of the dye from the cytosol. A Flexstation-3 microplate reader was used to measure basal calcium levels, carry out the addition of ligand samples to cells and to measure the subsequent intracellular calcium release response. Basal intracellular calcium levels were measured for 20 seconds, after which time the ligand compounds were added to the wells at predetermined concentrations and calcium levels were monitored for an additional 120 seconds. Calcium levels were measured in Relative Fluorescence Units (RFUs) throughout the time course. To determine Absolute RFUs, the basal RFU measured was deducted from the peak RFU for each sample (Absolute RFUs = maximum – minimum). ΔRFUs were obtained by deducting the Absolute RFU values of control WT-HEK293T

cells from those of T2R4-Gα16/44 expressing cells (ΔRFUs = Absolute RFUs of mutants – Absolute RFUs of WT). The experiments were repeated 4 times in triplicate.

Statistical Analysis.

Statistical analysis was carried out in PRISM 7.0. EC_{50} and IC_{50} values were unable to be calculated due to the limited number of concentrations assayed for dose response competition calcium assays. To analyze the statistical significance of error bars in bar plot (Figure 3), one-way analysis of variance was performed.

RESULTS

Docking of AGEs to T2R4.

A previously published T2R4 model constructed using the ITASSER server was used for ligand docking simulations due to its established validity through experiments with known agonists and antagonists *in vitro*.[19, 23] In addition to quinine, and bis-CML, and 23 AGEs were attempted to be docked into a 14.9Å sphere defined by the quinine binding pocket of T2R4 using the CDOCKER algorithm as described under "Experimental Procedures". Of the 25 compounds for which docking to T2R4 was attempted, crosslines was the only putative ligand that failed to dock. Comparison of docked ligands using a rank-by-rank strategy with seven different empirical scoring functions yielded a final ranking (Table 1). Also reported is a predicted binding affinity of each putative ligand based on the calculated Ludi Energy Estimate 3 score of each of the top poses, as mentioned in the methods section. This ranking was used to inform a selection of AGE compounds that would have their interaction capability with T2R4 tested *in vitro*, and GOLD and CML were selected as compounds to further characterize through functional studies.

Functional Characterization of GOLD and CML with T2R4.

In order to characterize the functional interaction of GOLD and CML with T2R4, changes in intracellular calcium levels were measured in T2R4-expressing HEK293T cells and HEK293T cells (control) in response to exposure of GOLD and CML. Quinine, a known agonist of T2R4 was used as a positive control, and applied to cells at a concentration of 1mM. To evaluate the ability of GOLD and CML to act as T2R4 agonists, these ligands were individually applied to both T2R4 expressing cell line and the control HEK293T cells in varying concentrations. Calcium mobilization response was minimal in response to either GOLD or CML treatment, suggesting that these compounds carried little if any agonistic activity with respect to T2R4 (Figure 2).

Given the limited calcium mobilization in T2R4-expressing cells over that of WT cells in response to treatment with either GOLD or CML, the potential of these compounds to act as T2R4 antagonists was investigated by way of a competitive assay with a known T2R4 agonist, quinine. Preliminary competition assays were carried out by the addition of GOLD and CML at varied concentrations (1 – 100 μM) in the presence of quinine fixed at its EC_{50} concentration (1 mM). Results of four repeated experiments carried out in triplicate reveal no statistically significant activity of GOLD nor CML to decrease the calcium mobilization response in quinine-treated T2R4-expressing mutants (Figure 3).

DISCUSSION

GPCRs represent a class of protein receptors of significant importance to the field of pharmaceuticals. They not only represent the largest human superfamily of receptors, and with their potential to trigger intracellular actions from extracellular signals while

comprising the largest human superfamily of receptors, it is not surprising to note that an estimated 50% of available drugs target GPCRs.[24]

T2Rs represent a subset of this protein superfamily that as of yet remains largely unexplored. They mediate the sensation of bitter taste in humans via the binding of small-molecule ligands referred to as tastants dissolved in saliva in the oral cavity. T2Rs are expressed in a variety of extraoral tissues including respiratory and gastrointestinal tissues.[4-9] The function of these T2Rs expressed in extraoral tissues is the subject of ongoing investigations. However, some recent studies have suggested a variety of potential pathophysiological roles for these receptors outside the oral cavity, which makes them intriguing potential drug targets.[9] While several hundred bitter tasting compounds have been reported in the literature, and about 100 agonists have been experimentally characterized for the 25 human T2Rs,[25] very few antagonists or bitter blockers have been identified.[26] Furthermore, none of the identified bitter blockers are capable of antagonizing all 25 human T2Rs.[26] Based on the previous identification of a synthetic amino acid derivative similar in structure to AGEs as a potent antagonist of T2R4[19] we herein report the use of a molecular modeling guided workflow to identify potential bitter blockers among the AGE class of molecules.

Functional Characterization of AGEs with T2R4.

Molecular modeling presents an enticing alternative to structure function studies guided by high-resolution structures in the field of membrane proteins such as GPCRs, the experimentally determined structures of which have remained largely elusive. The rationale for using the inactive rhodopsin crystal structure as a base template in the homology modeling protocol is that the T2R4 model that is built will have higher affinity

for antagonists, which is one of the principal objectives of this study. Virtual docking studies using this T2R4 model allowed the screening and ranking of a limited library of AGE compounds for their interaction potential with this bitter taste receptor (Table 1). Selected from among these compounds were GOLD and CML as promising targets for T2R4 interactivity based on commercial availability and rank-by-rank scoring. When cells expressing T2R4 were exposed to GOLD and CML during calcium mobilization assays, little calcium release was observed, thereby ruling out the role of these compounds as potent T2R4 agonists. Competition assays using of GOLD with the known T2R4 agonist quinine showed potential moderate levels of reduction in calcium release at increasing concentrations, while no such trend could be observed with the CML. It should be noted that this apparent downward trend in the calcium mobilization response of quinine-treated cells exists with increasing concentrations of GOLD is statistically non-significant with the currently available experimental data (Figure 3). Furthermore, due to statistical insignificance and the limited number of concentration variants used in dose-response competition assays, an IC_{50} value for GOLD could not be determined.

While the physiologic role of T2Rs in extraoral tissues expands the potential for the use of bitter blockers in theory to beyond the oral cavity, it is worthwhile to consider some factors necessary for the use of bitter blockers in food products as well. Previous studies have discussed that among other factors, a valuable bitter blocker to be used as a food additive should be safe for human consumption, should be functional in low doses, and ideally should be functional in blocking the full spectrum of 25 human T2Rs.[27] While GOLD and CML are endogenously produced compounds and therefore could conceivably be considered safe for human consumption with adequate testing, the results

described herein demonstrate that GOLD and CML fail to meet the two other important criteria. Indeed, no statistically significant inhibitory effect on the agonistic activity of Quinine on T2R4 was evident when GOLD and CML were applied at concentrations up to one-tenth that of the agonist (Figure 3). While it should be noted that there may be some value in testing the effect of GOLD and CML on quinine-dependent activation of T2R4 at higher concentrations, the use of these AGE compounds may present both technical and conceptual challenges in this respect. Increasing the concentration of the test compounds too greatly could lead to their precipitation out of aqueous solution due to low solubility, and high concentrations may negate physiologic relevance.

It may be expected that docking of ligands into a binding site in the T2R4 model that was generated based on previously identified residues that have been shown to be essential to quinine-mediated activation of T2R4 would lead to the identification of ligands that share the same interacting residues, and therefore have the potential to act as competitive inhibitors with quinine. Indeed, in the top scoring predicted pose for GOLD within the T2R4 binding pocket, several purported interactions are shared with quinine (Figure 4). Residues previously reported to be essential to quinine binding include Asn-173, Thr-174 and Tyr-258, while residues previously determined to be important to binding amino acid derived bitter blockers included Lys-270.[19] The top predicted pose for GOLD indicates certain commonalities with residues previously reported to be involved in quinine binding, through hydrogen bond donors from the backbone amine groups of Asn-173 and Thr-174 to a recipient on one of GOLD's two terminal hydroxyl groups. Furthermore, an additional common predicted interaction exists with the hydroxyl group from the side chain of Tyr-258 acting as a hydrogen bond donor to

GOLD's opposite terminal hydroxyl group. Interestingly, Lys-270 appears to play what would seem to be a crucial role in stabilizing GOLD within the binding pocket of T2R4, as its sidechain amino group contributes to a hydrogen bonding network with both of GOLD's terminal carbonyl groups.

In the case of CML, predicted hydrogen bond interactions again include residues essential for quinine binding (Asn-173, Thr-174 and Tyr-258), in addition to predicted hydrogen bond involvement with Gln-160, Arg-171, Asn-172, and Gln-257 and Lys-270 (Figure 4). It is interesting to note the role of Lys-270 again in the context of predicted CML binding, as the sidechain amine of this residue is involved in charge interactions with both of CML's terminal hydroxyl groups. The predicted involvement of backbone amine groups from the alpha helical residues of Asn-173 and Thr-174 is apparent as well. This reinforces the likely role of the essential structural contribution of these amino acid residues in the T2R4 ligand-binding site.

Future Directions.

Previous studies have demonstrated significant utility in the use of mutational studies to elucidate the quinine binding pocket of T2R4, and the roles played by residues therein.[(19)] In the event that statistically relevant data were to become available suggesting the GOLD carries the ability to inhibit T2R4 activation by quinine at higher concentrations, future investigations would necessarily turn toward mutational studies guided by the aforementioned molecular modeling-based interactions to determine the residues most necessary to allow GOLD's inhibitory action. Mutations would include both conserved and non-conserved point mutations (replacement of wild-type residues with those of similar and dissimilar functional groups respectively), to investigate the role

of predicted critical hydrogen bond and charge interactions in the binding pocket, in addition to the replacement of key residues with those of altered steric contribution to the binding pocket. Molecular modeling could then be employed once again in an effort to infer the origin of any experimentally validated residues with significant roles in T2R4 inhibition or activation. Such studies would further contribute to the current understanding of the structural role in the mechanism of T2R antagonism, and may serve well to inform on the inhibition of similar receptors from the greater GPCR superfamily.

It should also be noted that while neither GOLD nor CML would seem to act as highly potent inhibitors of quinine activation of T2R4 with the currently available data, it may be pertinent to investigate the potential role of cross-inhibition with other known T2R4 agonists such as yohimbine.[23] As quinine is currently the only T2R4 agonist with its binding pocket experimentally characterized, additional findings guided by molecular modeling regarding residues important for the action of other T2R4 agonists may well yield additional insights into the extents and specificity of the T2R4 agonist binding pocket, and essential factors involved in the activation mechanism of this receptor.

In conclusion, the present study has demonstrated the utility of a molecular modeling-based framework for the identification of potential AGEs that may act as agonists or antagonists on T2Rs. The use of molecular modeling allows the circumvention of significant challenges involved in high resolution structure determination of membrane proteins such as T2Rs, and allows for the rapid screening of a library of eligible compounds prior to commencing *in vitro* assays. From a set of 23 novel potential ligands for T2R4, GOLD and CML were chosen for further characterization by way of calcium mobilization and competition assays. Based on the

calcium assays performed, neither GOLD nor CML display appreciable activating or inhibitory activity on T2R4.

References

1. Hoon, M. A., Adler, E., Lindemeier, J., Battey, J. F., Ryba, N. J., and Zuker, C. S. (1999) Putative mammalian taste receptors: a class of taste-specific GPCRs with distinct topographic selectivity, *Cell 96*, 541-551.
2. Stewart, R. E., DeSimone, J. A., and Hill, D. L. (1997) New perspectives in a gustatory physiology: transduction, development, and plasticity, *The American journal of physiology 272*, C1-26.
3. Glendinning, J. I. (1994) Is the bitter rejection response always adaptive?, *Physiology & behavior 56*, 1217-1227.
4. Chandrashekar, J., Hoon, M. A., Ryba, N. J., and Zuker, C. S. (2006) The receptors and cells for mammalian taste, *Nature 444*, 288-294.
5. Chandrashekar, J., Mueller, K. L., Hoon, M. A., Adler, E., Feng, L., Guo, W., Zuker, C. S., and Ryba, N. J. (2000) T2Rs function as bitter taste receptors, *Cell 100*, 703-711.
6. Clark, A. A., Liggett, S. B., and Munger, S. D. (2012) Extraoral bitter taste receptors as mediators of off-target drug effects, *FASEB journal : official publication of the Federation of American Societies for Experimental Biology 26*, 4827-4831.
7. Behrens, M., and Meyerhof, W. (2010) Oral and extraoral bitter taste receptors, *Results and problems in cell differentiation 52*, 87-99.
8. Behrens, M., and Meyerhof, W. (2011) Gustatory and extragustatory functions of mammalian taste receptors, *Physiology & behavior 105*, 4-13.
9. Shaik, F. A., Singh, N., Arakawa, M., Duan, K., Bhullar, R. P., and Chelikani, P. (2016) Bitter taste receptors: Extraoral roles in pathophysiology, *The international journal of biochemistry & cell biology 77*, 197-204.
10. Uribarri, J., del Castillo, M. D., de la Maza, M. P., Filip, R., Gugliucci, A., Luevano-Contreras, C., Macias-Cervantes, M. H., Markowicz Bastos, D. H., Medrano, A., Menini, T., Portero-Otin, M., Rojas, A., Sampaio, G. R., Wrobel, K., Wrobel, K., and Garay-Sevilla, M. E. (2015) Dietary advanced glycation end products and their role in health and disease, *Advances in nutrition 6*, 461-473.
11. Singh, R., Barden, A., Mori, T., and Beilin, L. (2001) Advanced glycation end-products: a review, *Diabetologia 44*, 129-146.
12. Costanzi, S. (2013) Modeling G protein-coupled receptors and their interactions with ligands, *Current opinion in structural biology 23*, 185-190.
13. Kobilka, B., and Schertler, G. F. (2008) New G-protein-coupled receptor crystal structures: insights and limitations, *Trends in pharmacological sciences 29*, 79-83.
14. Cherezov, V., Rosenbaum, D. M., Hanson, M. A., Rasmussen, S. G., Thian, F. S., Kobilka, T. S., Choi, H. J., Kuhn, P., Weis, W. I., Kobilka, B. K., and Stevens, R. C. (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor, *Science 318*, 1258-1265.
15. Rasmussen, S. G., Choi, H. J., Rosenbaum, D. M., Kobilka, T. S., Thian, F. S., Edwards, P. C., Burghammer, M., Ratnala, V. R., Sanishvili, R., Fischetti, R. F.,

Schertler, G. F., Weis, W. I., and Kobilka, B. K. (2007) Crystal structure of the human beta2 adrenergic G-protein-coupled receptor, *Nature 450*, 383-387.
16. Rosenbaum, D. M., Cherezov, V., Hanson, M. A., Rasmussen, S. G., Thian, F. S., Kobilka, T. S., Choi, H. J., Yao, X. J., Weis, W. I., Stevens, R. C., and Kobilka, B. K. (2007) GPCR engineering yields high-resolution structural insights into beta2-adrenergic receptor function, *Science 318*, 1266-1273.
17. Mobarec, J. C., Sanchez, R., and Filizola, M. (2009) Modern homology modeling of G-protein coupled receptors: which structural template to use?, *Journal of medicinal chemistry 52*, 5207-5216.
18. Laskowski, R. A., Macarthur, M. W., Moss, D. S., Thornton, J. M. (1993) PROCHECK: a program to check the stereochemical quality of protein structures, *J. Appl. Cryst. 26*, 283-291.
19. Pydi, S. P., Sobotkiewicz, T., Billakanti, R., Bhullar, R. P., Loewen, M. C., and Chelikani, P. (2014) Amino acid derivatives as bitter taste receptor (T2R) blockers, *The Journal of biological chemistry 289*, 25054-25066.
20. Wang, R., Lu, Y., and Wang, S. (2003) Comparative evaluation of 11 scoring functions for molecular docking, *Journal of medicinal chemistry 46*, 2287-2303.
21. Kumar, M., Pydi, S. P., Sharma, S., Singh, T. P., and Kaur, P. (2014) Identification of a high affinity selective inhibitor of Polo-like kinase 1 for cancer chemotherapy by computational approach, *Journal of molecular graphics & modelling 51*, 104-112.
22. Chakraborty, R., Xu, B., Bhullar, R. P., and Chelikani, P. (2015) Expression of G protein-coupled receptors in Mammalian cells, *Methods in enzymology 556*, 267-281.
23. Pydi, S. P., Jaggupilli, A., Nelson, K. M., Abrams, S. R., Bhullar, R. P., Loewen, M. C., and Chelikani, P. (2015) Abscisic Acid Acts as a Blocker of the Bitter Taste G Protein-Coupled Receptor T2R4, *Biochemistry 54*, 2622-2631.
24. Tautermann, C. S. (2014) GPCR structures in drug design, emerging opportunities with new structures, *Bioorganic & medicinal chemistry letters 24*, 4073-4079.
25. Wiener, A., Shudler, M., Levit, A., and Niv, M. Y. (2012) BitterDB: a database of bitter compounds, *Nucleic acids research 40*, D413-419.
26. Jaggupilli, A., Howard, R., Upadhyaya, J. D., Bhullar, R. P., and Chelikani, P. (2016) Bitter taste receptors: Novel insights into the biochemistry and pharmacology, *The international journal of biochemistry & cell biology*.
27. Roland, W. S., Gouka, R. J., Gruppen, H., Driesse, M., van Buren, L., Smit, G., and Vincken, J. P. (2014) 6-methoxyflavanones as bitter taste receptor blockers for hTAS2R39, *PloS one 9*, e94451.

APPENDICES

FIGURE LEGENDS

Figure 1. Experimental framework for the identification and validation of novel T2R4 ligands. BIOVIA Discovery Studio was used to integrate both homology modeling and virtual ligand library generation. The results of the ligand screen were used to inform selection of two target ligands for further characterization *in vitro*. Molecular modeling also allows the assessment and interpretation of the results of *in vitro* studies according to predicted interactions in the model space, enabling subsequent targeted mutational analyses to be pursued.

Figure 2. Representative calcium traces. Cells stably expressing T2R4 and Gα16/44 were exposed to quinine, GOLD and CML, and subsequent changes in intracellular calcium levels were measured using Fluo-4 NW dye and a Flexstation 3 plate reader. Compounds were added to cells at the 20s time point and the change in intracellular calcium release in relative fluorescent units (RFU's) was measured for an additional 100s. ***A***, 100µM GOLD added to HEK293T cells expressing T2R4. ***B***, 100µM CML added to HEK293T cells expressing T2R4. ***C***, Quinine (1mM) added to HEK293T cells expressing T2R4. ***D***, Quinine (1mM) and GOLD (100µM) added to HEK293T cells expressing T2R4.

Figure 3. Summary of *in vitro* characterization of interaction of GOLD and CML with T2R4. Calcium assays were carried out using HEK293T cell line stably expressing T2R4 and Gα16/44, and with HEK293T cells as a negative control. Intracellular calcium release following the introduction of putative ligands to the wells was measured via fluorescence of calcium-binding dye Fluo-4 NW in Relative Fluorescent Units by a FlexStation-3 miniplate reader. Test compounds were added to the cell lines 20s after the start of fluorescence recording, and the total time course for each trial was 120s. Data was collected from four experiments carried out in triplicate. The basal fluorescent signal was subtracted from peak RFUs for each trial to yield a value of Absolute RFUs. Absolute RFUs from the negative control cell line was subtracted from that of the T2R4-Gα16/44 expressing cell line in each respective combination of compound exposure in order to yield ΔRFUs, as reported here. Compounds assayed included quinine (1mM), GOLD (1, 10 and 100µM) and CML (1, 10 and 100µM). While T2R4 expressing cells exhibited significantly increased intracellular calcium release as a result of exposure to quinine over that of HEK293T cells, neither GOLD nor CML exhibited any significant positive affect at the

concentrations assayed. When GOLD and CML were independently added to both test cell lines in the presence of quinine, a trend towards increasing quinine inhibition with increasing concentration of GOLD can be noted, however this is not statistically significant.

Figure 4. Predicted T2R4 interactions with GOLD and CML. Essential residues for quinine binding were used to generate a binding sphere (green) into which a library of AGE compounds were docked using BIOVIA Discovery Studio. Ligands were fit to the binding site using the CDOCKER protocol, and the top scoring pose for each compound was predicted using a rank-by-rank strategy that employed seven scoring functions. ***A,*** Schematic representation of T2R4 model and generated binding sphere utilized for ligand docking. ***B,*** Representation of top scoring docked pose for GOLD (green). ***C,*** Schematic representation of predicted critical interactions between GOLD and T2R4 residues. ***D,*** Representation of top scoring docked pose for CML (orange). ***E,*** Schematic representation of predicted critical interactions between CML and T2R4 residues.

Table 1. Compound library screen. A virtual library of AGE compounds was prepared by either modeling with PubChem Sketcher or by accessing directly from the PubChem database, wherever possible. 23 AGE compounds were docked with a previously published T2R4 model using the CDOCKER Protocol in Discovery Studio. Quinine, a T2R4 agonist and bis-CML, a previously characterized inverse agonist, were included as controls. Only one compound crosslines did not dock using this protocol. The top ten poses saved by CDOCKER were scored with seven scoring functions and ranked using a rank-by-rank strategy to inform choices for compounds that would be evaluated for T2R4 binding activity *in vitro*. A predicted K_d was derived using the Ludi Energy Estimate 3 score for each ligand, with the formula Ludi3 = $-100\log K_d$.

FIGURE 1.

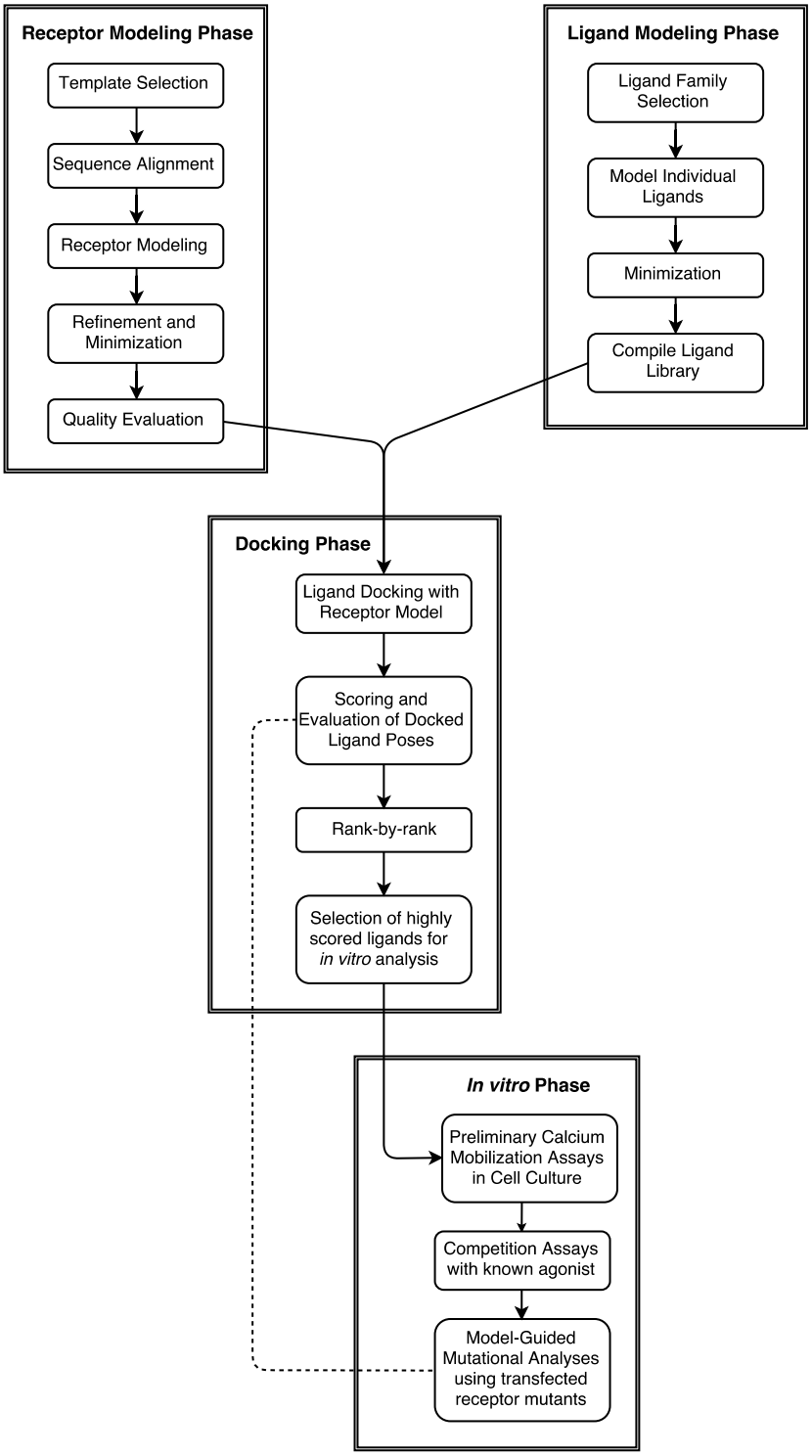
Receptor Modeling Phase
Template Selection
Sequence Alignment
Receptor Modeling
Refinement and Minimization
Quality Evaluation
Ligand Modeling Phase
Ligand Family Selection
Model Individual Ligands
Minimization
Compile Ligand Library
Docking Phase
Ligand Docking with Receptor Model
Scoring and Evaluation of Docked Ligand Poses
Rank-by-rank
Selection of highly scored ligands for *in vitro* analysis
In vitro Phase
Preliminary Calcium Mobilization Assays in Cell Culture
Competition Assays with known agonist
Model-Guided Mutational Analyses using transfected receptor mutants

FIGURE 2.

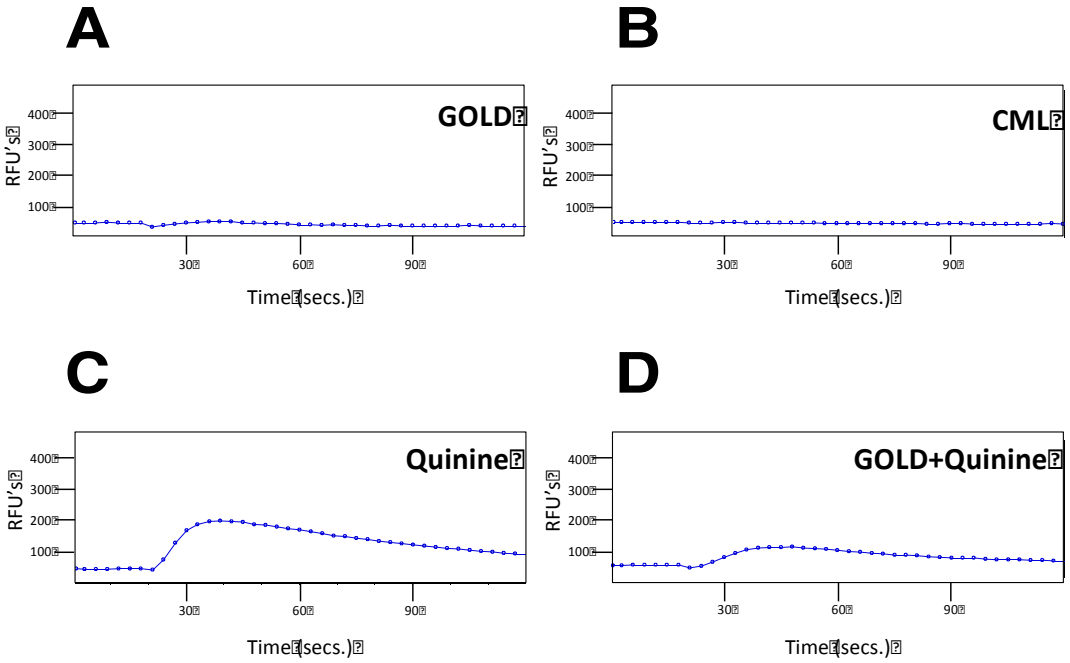
A
GOLD
RFU's
400
300
200
100
30
60
90
Time (secs.)
B
CML
RFU's
400
300
200
100
30
60
90
Time (secs.)
C
Quinine
RFU's
400
300
200
100
30
60
90
Time (secs.)
D
GOLD+Quinine
RFU's
400
300
200
100
30
60
90
Time (secs.)

FIGURE 3.

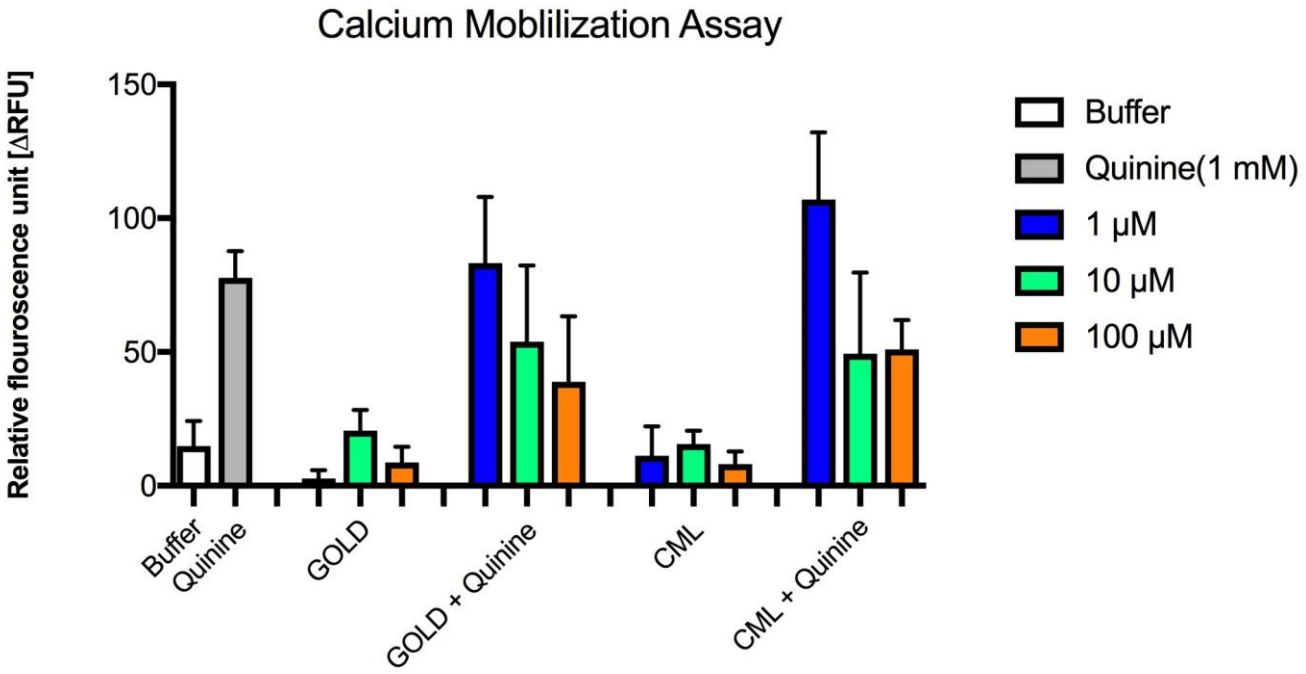
Calcium Moblilization Assay
Relative flouroscence unit [ΔRFU]
150
100
50
0
Buffer
Quinine
GOLD
GOLD + Quinine
CML
CML + Quinine
Buffer
Quinine(1 mM)
1 μM
10 μM
100 μM

FIGURE 4.

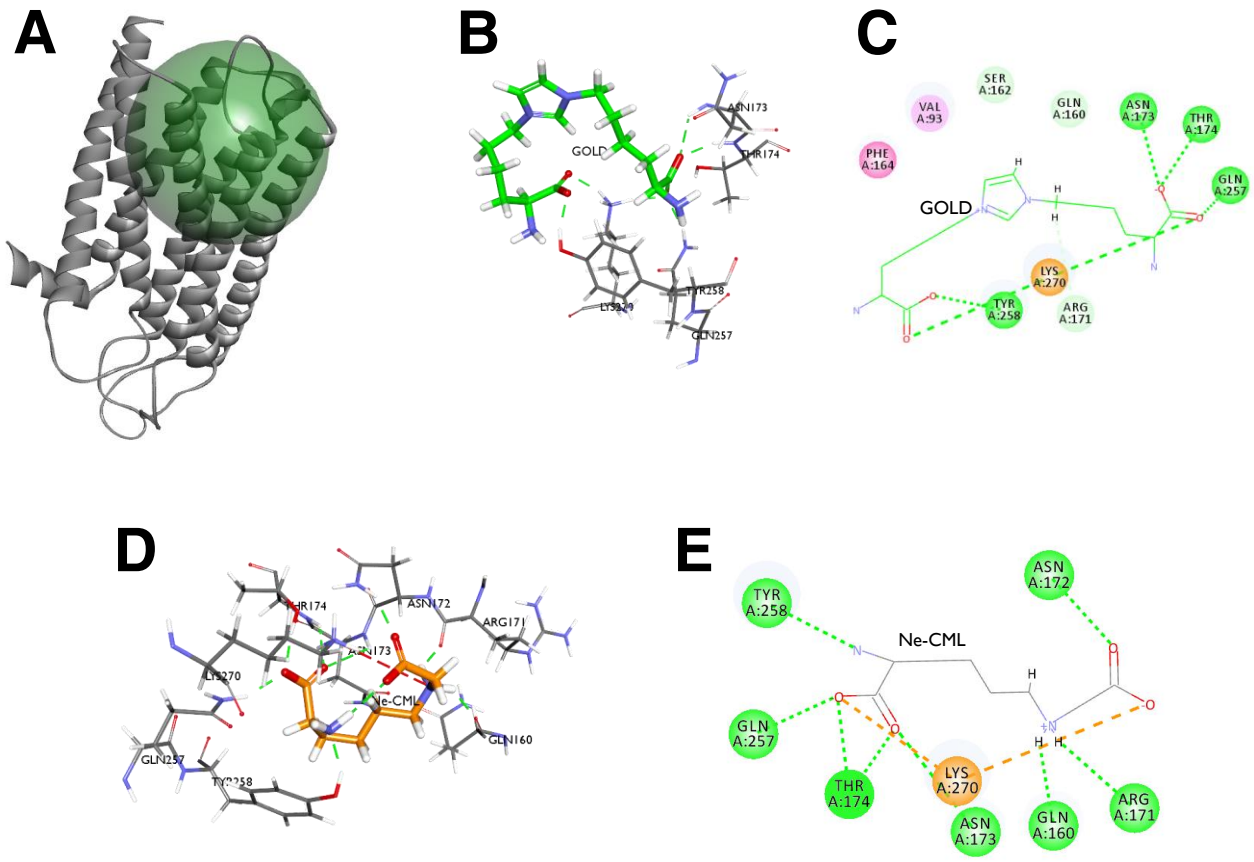
A
B
GOLD
ASN173
THR174
TYR258
GLN257
C
SER A:162
VAL A:93
GLN A:160
ASN A:173
THR A:174
PHE A:164
GLN A:257
GOLD
LYS A:270
TYR A:258
ARG A:171
D
THR174
ASN172
ARG171
ASN173
LYS270
Ne-CML
GLN160
GLN257
TYR258
E
TYR A:258
ASN A:172
Ne-CML
GLN A:257
THR A:174
LYS A:270
ASN A:173
GLN A:160
ARG A:171

TABLE 1.

Rank	Ligand	Predicted K_d Value (μM)
1	3-deoxyglucosone-derived lysine dimer (DOLD)	0.0741
2	1-Alkyl-2-formyl-3,4-glycosyl-pyrrole (AFGP)	2.1878
3	Tetrahydropyrimidine (THP)	0.0501
4	Pentosidine	0.0195
5	Glucosepane	1.2023
6	**Glyoxal-derived lysine dimer (GOLD)**	0.1096
7	3-deoxyglucosone hydroimidazolone 3 (3DG-H3)	0.309
8	Nε-carboxymethyl-hydroxylysine (CMhL)	0.6166
9	Methyl glyoxal hydroimidazolone 1(MG-H1)	0.1698
10	Fructosyl Lysine	0.3388
11	Pyrraline	0.6026
12	3-deoxyglucosone hydroimidazolone 1 (3DG-H1)	16.9824
12	Argpyrimidine	0.1549
14	3-deoxyglucosone hydroimidazolone 2 (3DG-H2)	1.6596
15	**Nε-Carboxymethyl-lysine (CML)**	0.0457
16	ImidazoloneA	1.9055
17	Methyl glyoxal-derived lysine dimer (MOLD)	0.2512
18	ImidazoloneB	5.1286
19	Nε-Carboxyethyl Lysine (CEL)	0.0912
20	Nα,Nα-bis(carboxymethyl)-L-lysine (bis-CML)	0.8913
21	Methyl glyoxal hydroimidazolone 2 (MG-H2)	2.3988
22	Glyoxal-derived hydroimidazolone (G-H)	19.9526
23	Methyl glyoxal hydroimidazolone 3 (MG-H3)	64.5654
24	Quinine	2.7542
Did not dock	Crosslines	Did not dock