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Article

Developing anticholinergic drugs for the treatment of asthma with improved efficacy

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SUMMARY

Anticholinergics are used in treating asthma, a chronic inflammation of the airways. These drugs block human M1 and M2 muscarinic acetylcholine receptors, inhibiting bronchoconstriction. However, studies have reported complications of anticholinergic usage, such as exacerbated eosinophil production and worsened urinary retention. Modification of known anticholinergics using bioisosteric replacements to increase efficacy could potentially minimize these complications. The present study focuses on identifying viable analogs of anticholinergics to improve binding energy to the receptors compared to current treatment options. Glycopyrrolate (G), ipratropium (IB), and tiotropium bromide (TB) were chosen as parent drugs of interest, due to the presence of common functional groups within the molecules, specifically esters and alcohols. Docking score analysis via AutoDock Vina was used to evaluate the binding energy between drug analogs and the muscarinic acetylcholine receptors. The final results suggest that G-A3, IB-A3, and TB-A1 are the most viable analogs, as binding energy was improved when compared to the parent drug. G-A4, IB-A4, IB-A5, TB-A3, and TB-A4 are also potential candidates, although there were slight regressions in binding energy to both muscarinic receptors for these analogs. By researching the effects of bioisosteric replacements of current anticholinergics, it is evident that there is a potential to provide asthmatics with more effective treatment options.

INTRODUCTION

Characterized by airway obstruction and wheezing due to inflammation of the airways, asthma is a chronic respiratory condition marked by spasms in the bronchi. Asthma exacerbations typically result from an allergic reaction or other forms of hypersensitivity. Symptoms may include frequent coughs, chest pain, wheezing, or difficulty breathing. Inhalers are often used by asthmatics to relieve the contraction of smooth muscle bands surrounding the bronchial airways (1).

Studies have suggested a correlation between the neural control of airways in asthma and neurocentric mechanisms (2). Within the bronchoconstriction pathway, cholinergic nerves may lead to increased cholinergic neurotransmission due to the effects of inflammatory mediators in patients with asthma (2). Upon stimulation, cholinergic nerves cause bronchoconstriction and promote the activity of the submucosal gland (2). Anticholinergic drugs are used for the treatment of asthma and cause the airways to widen by blocking these cholinergic nerves (3). They begin to work in short periods, typically fifteen minutes after inhalation or ingestion, and usually persist three to six hours in the human body (4).

Previous studies have revealed complications associated with the use of anticholinergic drugs as a treatment for asthma, including side effects such as headaches and nausea as well as increased risks for the neurovascular system, especially heart palpitations and tachycardia (5). For example, ipratropium bromide ($C_{20}H_{30}BrNO_3$), a common anticholinergic drug, has been found to exacerbate the production of eosinophils, white blood cells that promote inflammation, which are usually absent in the central nervous system of healthy individuals (6). Issues with current anticholinergic drugs and the limited library of anticholinergic compounds have slowed the progress of finding effective anticholinergic drugs (7). Modification of known anticholinergics presents an avenue of drug design exploration that may overcome current risk profiles (7).

Therefore, we sought out the drug structures of three known anticholinergic drugs, glycopyrrolate (G) ($C_{19}H_{28}BrNO_3$), ipratropium bromide (IB) (C20H30BrNO3), and tiotropium bromide (TB) ($C_{19}H_{22}BrNO_4S_2$). We selected these specific anticholinergic drugs because they are synthetic quaternary ammonium congeners of atropine, which are very poorly absorbed when given by inhalation (7). As these parent drugs have been widely used in combination with beta-adrenergic agents such as Albuterol for acute exacerbations of airway obstruction, we chose to identify viable analogs of these known anticholinergics (4).

Since each anticholinergic drug shares common functional groups, specifically an ester and alcohol, these groups were targeted for modulation. By replacing common functional groups with known bioisosteres, we constructed a library of analogs to analyze the binding energy and interactions between the target models and parent drugs as a means to assess the performance of the generated analogs. Subsequently, we determined whether the analogs had improved binding energy with classical cholinergic targets.

Our analog library produced three most favorable candidates for future exploration: G-A3, IB-A3, and TB-A1.

Overall, all three candidates demonstrated improved binding energy with human muscarinic acetylcholine receptors, which translates to a potential increase in bioavailability, and these changes could metabolically stabilize the compounds for a longer period of therapeutic activity. Our efforts to increase the library of analogs for targeting acetylcholine receptors for treating asthma can provide novel small molecules with similar safety profiles to currently approved FDA drugs.

RESULTS

Parent drugs were modulated with different bioisosteres, targeting common functional groups and replacing them with functional groups that behave chemically similar. Bioisosteric substitutions were selected that are known to generally increase metabolic stability and bioavailability (13-15). This process generated five analogs for each parent drug. Upon generating the analogs, target receptors M1 (PDB-ID:5CXV) and M2 (PDB-ID:3UON) were identified. M1 and M2 are human muscarinic acetylcholine receptors, which play a key role in the neural pathway controlling asthma. M1 and M2 were specifically chosen as holistic models of the human muscarinic acetylcholine receptors. We performed preliminary studies on the inhibitory capacity of the analogs by analyzing their binding energy with M1 and M2.

After running docking simulations with each parent drug and their analogs with targets M1 and M2, we compiled each docking score (**Table 1**) (9-12). This created a baseline for binding energy in kcal/mol for each parent drug to gauge improvements or regressions in the binding energy of analogs. Negative scores correspond with improved binding energy, indicating a better analog, promoting inhibitory capacity (12). While comparing Autodock Vina's values for parent drugs and analogs, it is necessary to consider that Autodock Vina calculates the binding energy with a root mean square error of approximately 2.8 kcal/mol (12).

Docking Score		
Compounds	M1	M2
	5CXV (kcal/mol)	3UON (kcal/mol)
Glycopyrrolate	-6	-10.1
G-A1	-6.2	-10
G-A2	-6.1	-9
G-A3	-6.7	-10.6
G-A4	-6.5	-10.3
G-A5	-6.1	-8.9
Ipratropium bromide	-7.2	-9.2
IB-A1	-6.7	-8.8
IB-A2	-6.6	-8.8
IB-A3	-6.6	-9.8
IB-A4	-6.4	-9.9
IB-A5	-5.7	-9.6
Tiotropium bromide	-5.7	-9.8
TB-A1	-5.7	-9.8
TB-A2	-5.5	-8.1
TB-A3	-6	-9
TB-A4	-6	-8.9
TB-A5	-5.9	-7.7

Table 1: Top binding scores of parent drugs and their analogs to M1 and M2 receptors.

The active sites of M1 or M2 were modeled in complex with parent drugs or by the top five analogs based on binding affinity: glycopyrrolate ($C_{19}H_{28}BrNO_3$) (**Figure 1**); ipratropium bromide ($C_{20}H_{30}BrNO_3$) (**Figure 2**); and tiotropium bromide ($C_{19}H_{22}BrNO_4S_2$) (**Figure 3**). Each top analog bound with targets M1 or M2 in a similar conformation compared to its parent drug, suggesting similar inhibitory capacity.

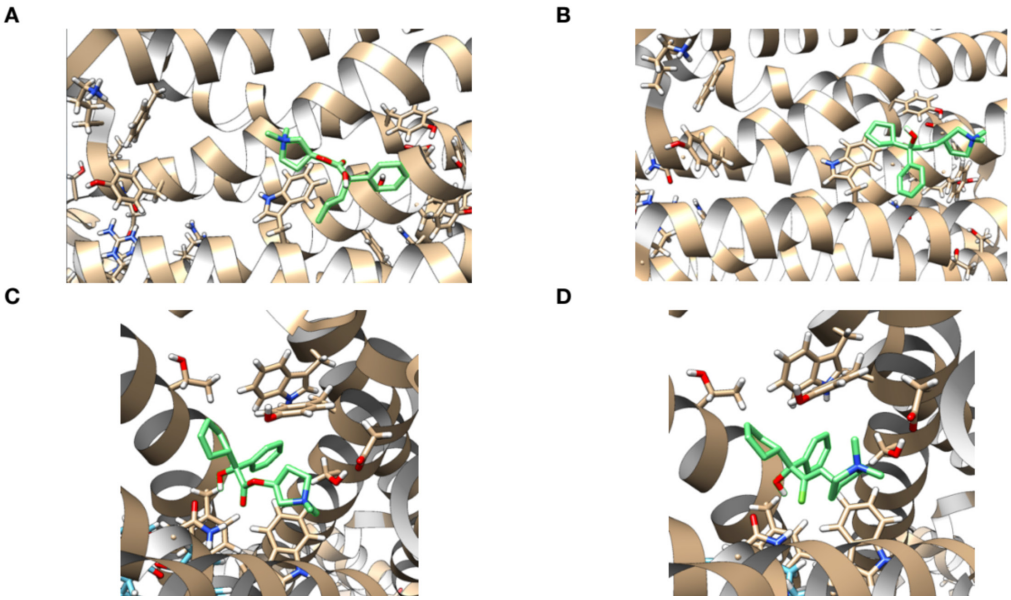

Figure 1: Models of glycopyrrolate and its analogs in the active site of human muscarinic acetylcholine receptors. (A) Glycopyrrolate bound to the active site of human M1 muscarinic acetylcholine receptor (5CXV). (B) Best performing analog of glycopyrrolate, G-A3, bound to the active site of human M1 muscarinic acetylcholine receptor (5CXV). (C) Glycopyrrolate bound to the active site of human M2 muscarinic acetylcholine receptor (3UON). (D) G-A3 bound to the active site of human M2 muscarinic acetylcholine receptor (3UON). For all images, the analog or parent drug is outlined in light green, with the tan protein and side chains constituting the active site in the surroundings.

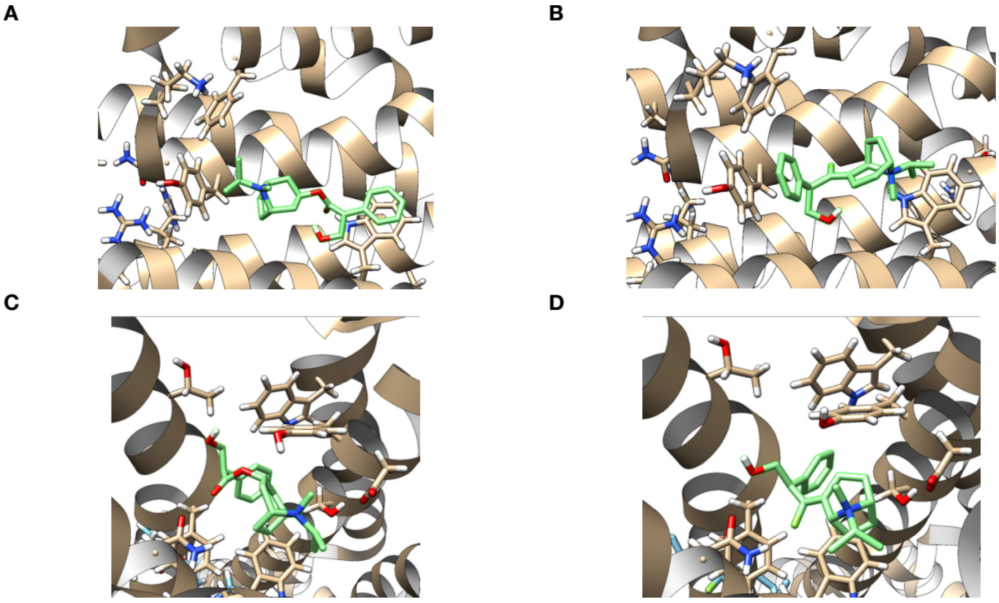

Figure 2: Models of ipratropium bromide and its analogs in the active site of human muscarinic acetylcholine receptors. (A) Ipratropium bromide bound to the active site of human M1 muscarinic acetylcholine receptor (5CXV). (B) Best performing analog of ipratropium bromide, IB-A3, bound to the active site of human M1 muscarinic acetylcholine receptor (5CXV). (C) Ipratropium bromide bound to the active site of human M2 muscarinic acetylcholine receptor (3UON). (D) IB-A3 bound to the active site of human M2 muscarinic acetylcholine receptor (3UON). For all images, the analog or parent drug is outlined in light green, with the tan protein and side chains constituting the active site in the surroundings.

Glycopyrrolate ($C_{19}H_{28}BrNO_3$) is abbreviated as G, ipratropium bromide ($C_{20}H_{30}BrNO_3$) as IB, and tiotropium bromide ($C_{19}H_{22}BrNO_4S_2$) as TB, with identifying suffixes ranging from A1-A5 detailing bioisosteric substitutions (**Table 2**). To create analogs of the parent drugs, we substituted the original functional groups for new groups. The following functional group swaps were explored: changing an alcohol to an amine (A1), changing an alcohol to a thiol (A2), changing from C=O to C-F and an alkene shift (A3); the haloalkane and alkene shift in conjunction with the amine (A4), and the haloalkane and alkene shift with the thiol (A5) (**Figure 4-6**).

Glycopyrrolate ($C_{19}H_{28}BrNO_3$) showed a baseline binding energy of -6.0 kcal/mol in complex with target M1 and -10.1 kcal/mol in complex with target M2. Of the five glycopyrrolate

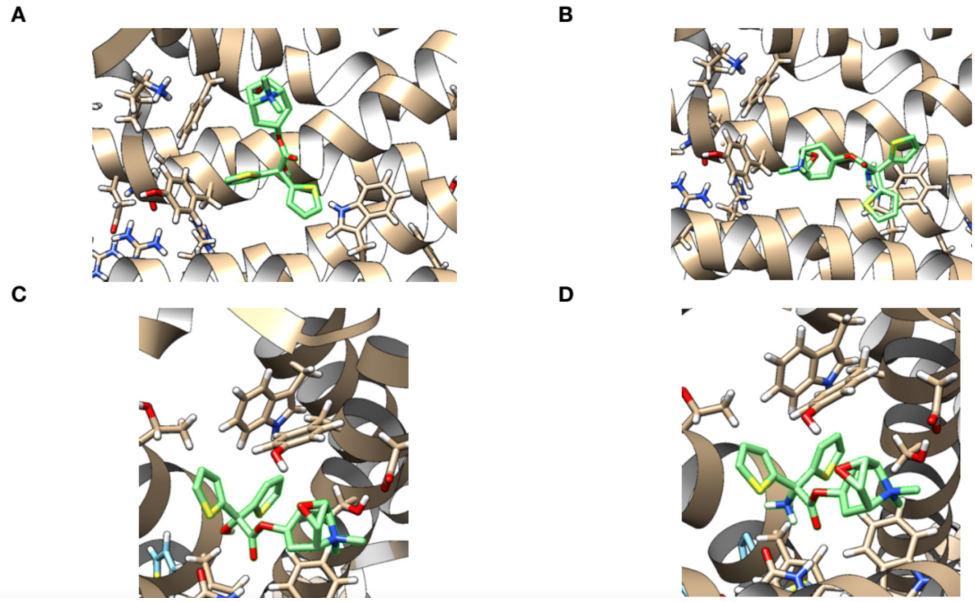

Figure 3: Models of tiotropium bromide and its analogs in the active site of human muscarinic acetylcholine receptors. (A) Tiotropium bromide bound to the active site of human M1 muscarinic acetylcholine receptor (5CXV). (B) Best performing analog of tiotropium bromide, TB-A1, bound to the active site of human M1 muscarinic acetylcholine receptor (5CXV). (C) Tiotropium bromide bound to the active site of human M2 muscarinic acetylcholine receptor (3UON). (D) Best performing analog of parent drug, TB-A1, bound to the active site of human M2 muscarinic acetylcholine receptor (3UON). For all images, the analog or parent drug is outlined in light green, with the tan protein and side chains constituting the active site in the surroundings.

Analog Suffix	Bioisostere Substitutions
A1	Substitution of alcohol with amine
A2	Substitution of alcohol with thiol
A3	Substitution of C=O with C-F, alkene shift
A4	Substitution of alcohol with amine and C=O with C-F, alkene shift
A5	Substitution of alcohol with thiol and C=O with C-F, alkene shift

Table 2: Description of analog suffixes and corresponding bioisostere substitutions.

($C_{19}H_{28}BrNO_3$) analogs in complex with M1, all analogs showed improvements in binding energy compared to glycopyrrolate, although G-A1, G-A2, a substitution of alcohol with thiol, and G-A5, a substitution of alcohol with thiol and C=O with C-F, were only slight improvements, ranging from -0.1 to -0.2 kcal/mol (**Figure 4**) (**Table 1**). Not all analogs had increased binding affinity with M2, unlike M1. When complexed with target M2, G-A3, a substitution of C=O with C-F, demonstrated an improvement in the binding energy of -0.5 kcal/mol. There was also a slight improvement for G-A4, a substitution of alcohol with amine and C=O with C-F, of -0.2 kcal/mol (**Figure 4**) (**Table 1**). Analog G-A1's binding energy slightly worsened from the baseline by 0.1 kcal/mol, whereas analogs G-A2 and G-A5 significantly weakened binding by 1.1 and 1.3 kcal/mol, respectively (**Figure 4**) (**Table 1**). Therefore, for glycopyrrolate analogs, we posit that the binding energy profile of G-A3 is the best for M1 and M2 receptors.

Ipratropium bromide ($C_{20}H_{30}BrNO_3$) exhibited a baseline binding energy of -7.2 kcal/mol when binding to target M1 and -9.2 kcal/mol with target M2. IB-A1, a substitution of alcohol with amine; IB-A2, a substitution of alcohol with thiol; and IB-A3, a substitution of C=O with C-F, demonstrated slight regressions when bound to M1, with deterioration being either 0.5 or 0.6 kcal/mol (**Figure 5**) (**Table 1**). IB-A4, a substitution of alcohol with amine and C=O with C-F; and IB-A5, a substitution of alcohol with thiol and C=O with C-F, were subject to significant decreases in binding energy with M1, worsening by 0.8 or 1.5 kcal/mol, respectively (**Figure 5**) (**Table 1**). When bound to M2, IB-A3, IB-A4, and IB-A5 improved in energy, rising by -0.6, -0.7, and -0.4 kcal/mol, respectively (**Figure 5**) (**Table 1**). However, IB-A1 and IB-A2 were found to decrease in binding energy by 0.6 kcal/mol (**Figure 5**) (**Table 1**). As such, we conclude that IB-A3 holds the best binding energy scores for M1 and M2 receptors of all ipratropium bromide analogs.

Tiotropium Bromide ($C_{19}H_{22}BrNO_4S_2$) experienced a baseline binding energy of -5.7 kcal/mol in complex with target M1, and -9.8 kcal/mol in complex with target M2 (**Table 1**). Of the five Tiotropium Bromide ($C_{19}H_{22}BrNO_4S_2$) analogs in complex with M1, TB-A3, a substitution of C=O with C-F; TB-A4, a substitution of alcohol with amine and C=O with C-F; and TB-A5, a substitution of alcohol with thiol and C=O with C-F experienced marginal increases in binding energy, varying from -0.2 to -0.3 kcal/mol, whereas TB-A1, a substitution of alcohol with amine, maintained the baseline; and TB-A2, a substitution of alcohol with thiol, slightly worsened by 0.2 kcal/mol (**Figure 6**) (**Table 1**). When complexed with M2, only TB-A1 maintained the baseline, whereas TB-A3 and TB-A4 slightly regressed by 0.8 or 0.9 kcal/mol, respectively; and TB-A2 and TB-A5 dramatically worsened by 1.7 and 2.1 kcal/mol, respectively (**Figure 6**) (**Table 1**). For tiotropium bromide analogs, it appears that TB-A1 has the best balance of binding energy interactions with M1 and M2 receptors.

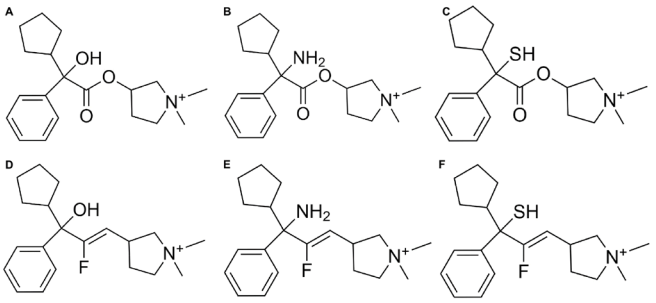

Figure 4: Chemical structures of parent drug glycopyrrolate and its analogs. (A) Chemical structure of parent drug glycopyrrolate (DB00986). (B) G-A1, bioisosteric replacement of alcohol with an amine functional group. (C) G-A2, bioisosteric substitution of alcohol with thiol group substitution. (D) G-A3, bioisosteric substitution of C=O with C-F and alkene shift. (E) G-A4, bioisosteric replacement of alcohol with an amine functional group and bioisosteric substitution of C=O with C-F and alkene shift. (F) G-A5, bioisosteric substitution of alcohol with thiol group substitution and bioisosteric substitution of C=O with C-F and alkene shift.

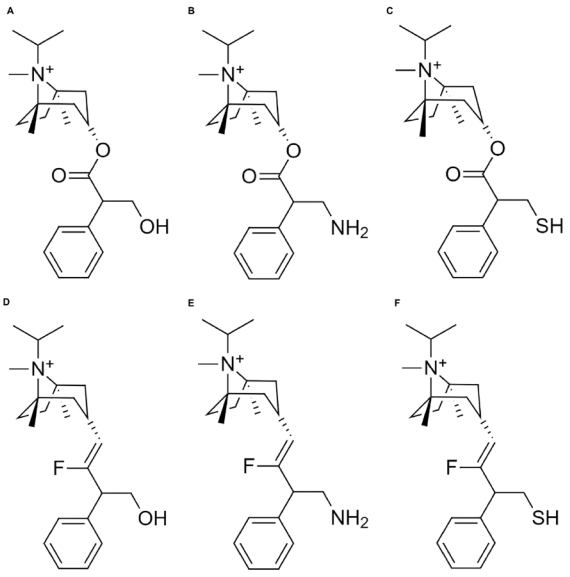

Figure 5: Chemical structures of parent drug ipratropium bromide and its analogs. (A) Chemical structure of parent drug ipratropium bromide (DB00332). (B) IB-A1, bioisosteric replacement of alcohol with an amine functional group. (C) IB-A2, bioisosteric substitution of alcohol with thiol group substitution. (D) IB-A3, bioisosteric substitution of C=O with C-F and alkene shift. (E) IB-A4, bioisosteric replacement of alcohol with an amine functional group and bioisosteric substitution of C=O with C-F and alkene shift. (F) IB-A5, bioisosteric substitution of alcohol with thiol group substitution and bioisosteric substitution of C=O with C-F and alkene shift.

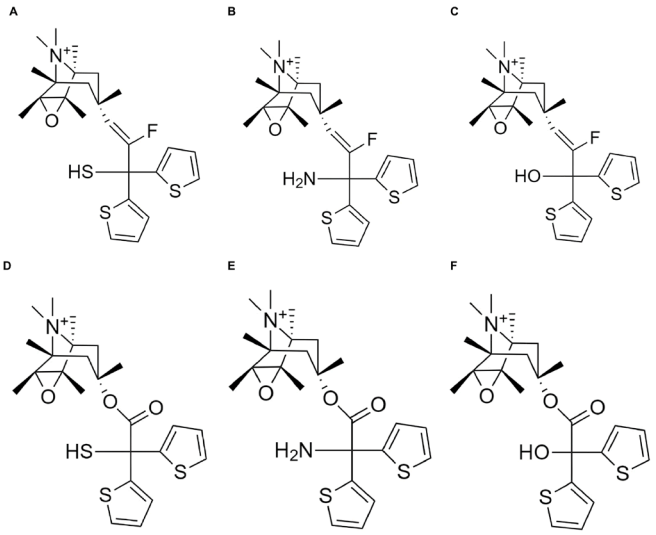

Figure 6: Chemical structures of parent drug tiotropium bromide and its analogs. (A) Chemical structure of parent drug tiotropium bromide (DB0140). (B) TB-A1, bioisosteric replacement of alcohol with an amine functional group. (C) TB-A2, bioisosteric substitution of alcohol with thiol group substitution. (D) TB-A3, bioisosteric substitution of C=O with C-F and alkene shift. (E) TB-A4, bioisosteric replacement of alcohol with an amine functional group and bioisosteric substitution of C=O with C-F and alkene shift. (F) TB-A5, bioisosteric substitution of alcohol with thiol group substitution and bioisosteric substitution of C=O with C-F and alkene shift.

DISCUSSION

Of the five glycopyrrolate ($C_{19}H_{28}BrNO_3$) analogs constructed and docked across the two targets, G-A3 was the highest performing analog for future testing as it exhibited the most improvement in binding to both targets. G-A4 performed similarly to G-A3 and can also be considered for future testing. The shared improvement for both G-A3 and G-A4 can likely be attributed to the fact that both analogs substituted their carbonyls with a haloalkane, specifically C-F, shifting the alkene to compensate. The difference between G-A3 and G-A4 can be attributed to the amine substitution in the latter, slightly decreasing performance. While less noteworthy, G-A1, G-A2, and G-A5 were considered as well for their improvement with human muscarinic receptors M1 despite their regression with M2. The bioisosteric substitutions present in G-A1, G-A2, and G-A5 may improve M1 selectivity rather than decrease overall performance.

After analyzing the docking scores of ipratropium bromide ($C_{20}H_{30}BrNO_3$) and its analogs, IB-A3 appeared to be superior to the others due to its greatest net improvement across M1 and M2. IB-A4 also seemed to be a viable option due to a similar net improvement. However, IB-A1, IB-A2, and IB-A5 exhibited more considerable regressions in binding energy and are unlikely to perform as well as IB-A3 and IB-A4. IB-A3 and IB-A4 are characterized by their shared substitution of a haloalkane and alkene shift over a carbonyl, with IB-A4 having an additional amine substitution. As glycopyrrolate ($C_{19}H_{28}BrNO_3$) and ipratropium bromide ($C_{20}H_{30}BrNO_3$) both display improved analogs A3 and A4, it may be worth further investigation of the inhibitory potential of the bioisosteric substitutions that comprise these analogs, most notably the carbonyl substitution.

Finally, of the tiotropium bromide ($C_{19}H_{22}BrNO_4S_2$) analogs, TB-A1 was the most viable from a computational perspective. While it did not exhibit any change in binding scores, the bioisosteric substitution of the amine group may enhance selectivity of TB-A1 with receptors by decreasing binding energy with off-targets. The amine substitution may also be beneficial in prolonging bioavailability through metabolic stability. TB-A3 and TB-A4 were also potentially viable analogs due to the improvement in binding energy with M1 despite a marginal decrease in energy with M2. They may also warrant exploration due to the computationally promising performance of A3 and A4 analogs in glycopyrrolate ($C_{19}H_{28}BrNO_3$) and ipratropium bromide ($C_{20}H_{30}BrNO_3$). However, TB-A2 and TB-A5 are unlikely to be noteworthy due to the dramatic decrease in binding energy with the M2 receptor.

To summarize, G-A3, IB-A3, and TB-A1 were the most favorable analogs in our constructed library. G-A3 demonstrated improved binding energy with human muscarinic acetylcholine receptors M1 and M2, which may potentially prolong bioavailability by replacing an easily cleavable bond (13-15). Similarly, IB-A3 experienced a net improvement in binding energy with M1 and M2 while likely improving bioavailability. Lastly, TB-A1 maintained binding energies while potentially improving metabolic stability through the substitution of an amine group over an alcohol group. A few other analogs may also be viable, including G-A4, IB-A4, TB-A3, and TB-A4, as they exhibited similar binding energies to the optimal analogs listed above, but further testing is required.

Potential areas for future study include the synthesis of each favorable analog and the *in vitro* analysis of these analogs in comparison to their parent drugs. The most notable comparisons to be made include testing for increased bioavailability (provided through improved metabolic stability or resistance to cleavage) and improved binding energy (13-15). Should the analysis and comparison be constructive, further *in vivo* testing or clinical trials could be conducted to assess the potential of these analogs as alternative compounds. Other investigations include computationally determining the extent to which the electronegativity of the parent drug, and subsequent analogs, affect the binding affinities of the ligand to the protein receptors analyzed in the present study. Previous studies on the structure relationship between neuroactive flavonoids and the GABA receptor found that the incorporation of highly electronegative groups on the ligand yields an increase in the binding affinity of the protein receptor site (16). Similarly, adding functional groups with high electronegativity, e.g. fluorine vs. oxygen, as is the case with G-A3 and IB-A3 may have contributed to enhanced binding of muscarinic receptors M1 and M2.

There are some restrictions to the current study. As it was purely computational, the difficulty and feasibility of synthesis were not considered when determining the most viable analogs. All predicted changes in bioavailability were solely based on previous findings and are untested with these analogs. Furthermore, docking analysis was only performed with one software and the ADME-T properties (absorption, distribution, metabolism, excretion, and toxicity) of each analog were not estimated. We intend to investigate and corroborate the computational findings here with other

binding prediction algorithms in the future.

In summary, to broaden the library of drug candidates in the anticholinergic field, we constructed a catalog of 15 analogs. Of these 15 analogs, we have identified G-A3, IB-A3, and TB-A1 as the most viable and have performed preliminary docking simulations with classical cholinergic targets, M1 and M2 receptors. G-A3 and IB-A3 are characterized by a substitution of C=O with C-F and an alkene shift, whereas TB-A1 is characterized by a substitution of an amine group for its alcohol group. By increasing the number of viable compounds for the anticholinergic treatment of asthma, we aim to begin improving the quality of life of asthmatics with new approaches.

MATERIALS AND METHODS

Drug Candidate Selection

Anticholinergic drugs were identified via a literature review. Potential candidates for modification included ipratropium bromide ($C_{20}H_{30}BrNO_3$), tiotropium bromide ($C_{19}H_{22}BrNO_4S_2$), oxitropium bromide ($C_{19}H_{26}BrNO_4$), glycopyrrolate ($C_{19}H_{28}BrNO_3$), aclidinium bromide ($C_{26}H_{30}BrNO_4S_2$), and umeclidinium bromide ($C_{29}H_{34}BrNO_2$) (17). Glycopyrrolate ($C_{19}H_{28}BrNO_3$), ipratropium bromide ($C_{20}H_{30}BrNO_3$), and tiotropium bromide ($C_{19}H_{22}BrNO_4S_2$) were selected for further study, as all three are synthetic quaternary ammonium congeners of atropine ($C_{17}H_{23}NO_3$) (4,8).

Drug Model Construction

Analyses were performed using Avogadro version 1.2.0 (18). Glycopyrrolate ($C_{19}H_{28}BrNO_3$), ipratropium bromide ($C_{20}H_{30}BrNO_3$), and tiotropium bromide ($C_{19}H_{22}BrNO_4S_2$) were constructed, referencing structures obtained from Drugbank (18,19). Structures were geometrically optimized according to Avogadro's Universal Force Field (UFF) Geometry Optimization protocol, based on work by Rappé *et al*. on the UFF (20). By using the UFF protocol to optimize molecular geometry, the ligand's atomic bond lengths, charges, angles, torsions, and interactions were accounted for in preparation for docking simulations. After structures were optimized, they were checked against Drugbank's structures to confirm stereochemistry.

Analog Development

As all selected parent drugs share common functional groups, such as an ester and alcohol, these groups were targeted for modulation. In order to improve the stability of analogs against metabolism within the body, the alcohol group was substituted with either an amine group or a thiol group (15). In an attempt to increase bioavailability, the easily cleavable C=O of the ester in the parent drug was replaced with a less reactive C-F functional group and alkene shift. The C=O of the ester was substituted with C-F and an alkene shift in order to replace an easily cleavable portion of the parent drug with a less reactive functional group in an attempt to increase bioavailability (13-15). Analog nomenclature has been simplified to acronyms for parent drug names in addition to analog suffixes, referring to the substituted functional group(s) with accompanying analog structures (**Table 2**) (**Figure 4-6**).

Target Selection

Upon constructing all parent drugs and analogs, drug targets were identified using Drugbank (21-24). The cholinergic receptors human muscarinic acetylcholine receptors M1 and M2 were relevant targets for all three parent drugs. Each receptor protein model was obtained from RCSB Protein Data Bank (M1: 5CXV, M2: 3UON) and active sites were identified based on ligand interactions with side chains (10,11). Side chain numbers were found through RCSB and relocated in UCSF Chimera (25).

Docking Analysis

The active sites of M1 and M2 were obtained by referencing the intermolecular forces between ligands and proteins in RCSB. Drug target models were imported into UCSF Chimera version 1.11.2 and ligands within receptors M1 and M2 were removed (25). Water (H2O) molecules were also removed (25). Parent drugs and analogs were imported into their relevant target models. Using Chimera's AutoDock Vina dialogue, the receptor search volume was generated by inputting randomized parameters to visualize the search volume (9,12). The search volume was subsequently refined by manually resizing it to encapsulate the protein's active site as closely as possible. Before running the simulation, it was confirmed that all active site side chains were confined within the search volume. Vina's default docking parameters were used when calculating binding energy. These default parameters include functions such as "remove non-polar hydrogens" and "remove lone pairs" to simplify calculations (9,12). Vina was used to calculate up to nine binding modes at its maximum exhaustiveness with a maximum energy difference of 3 kcal/mol. Then, docking simulations were performed to analyze the binding energy (12). The interactions between the protein receptor and either parent drugs or generated analogs were also analyzed.

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