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Bitterless Guaifenesin Prodrugs – Design, Synthesis, Characterization, In Vitro Kinetics and Bitterness Studies

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Abstract:

A respected number of drugs suffer from bitter taste which results in patient incompliance. With the aim of solving the bitterness of guaifenesin, dimethyl maleate, maleate, glutarate, succinate and dimethyl succinate prodrugs were designed and synthesized. Molecular orbital methods were utilized for the design of the ester prodrugs.

The density functional theory (DFT) calculations revealed that the hydrolysis efficiency of the synthesized prodrugs is significantly sensitive to the pattern of substitution on C=C bond and distance between the nucleophile and the electrophile. The hydrolysis of the prodrugs was largely affected by the pH of the medium. The experimental $t_{1/2}$for the hydrolysis of guaifenesin dimaleate ester prodrugs in 1N HCl was the least and for guaifenesin dimethyl succinate was the highest.

Functional heterologous expression of TAS2R14, a broadly tuned bitter taste receptor responding to guaifenesin, and experiments using these prodrugs revealed that, while some of the prodrugs still activated the receptor similarly or even stronger than the parent substance, succinate derivatization resulted in the complete loss of receptor responses**.** The predicted binding modes of guaifenesin and its prodrugs to the TAS2R14 homology model suggest that the decreased activity of the succinate derivatives may be caused by a clash with Phe247.

1. Introduction:

A significant number of prescribed and OTC (over-the-counter) drugs are bitter. The bitter and unpleasant taste of drugs is a major obstacle in formulating pediatrics and geriatrics drugs and is considered as a great challenge to the health community [1,2].The most commonly used bitter tasting drugs include paracetamol, ampicillin, azithromycin, diphenhydramine, erythromycin, ibuprofen, penicillin, pseudoephedrine and guaifenesin [3-5].Nowadays, the medicinal industry has recognized the importance of taste masking, however, the development of an appropriate formulation is relatively expensive and time consuming [6, 7].

A vast number of techniques have been invoked for concealing the unpleasant taste of drugs [8-10], including polymer coating, complex formation with β-cyclodextrin, ion exchange resins and solubility reducing methods. On the other hand, traditional taste masking techniques such as the use of sweeteners and flavoring agents, such as anise oil, cardamom, lemon and orange, or artificial flavors, alone resulted in inadequate masking of the taste of extremely bitter drugs. It is worth noting that excipients such as sweetening flavors, menthol and chloroform to anaesthetize taste receptors, have failed to mask 70% of the tested drugs. Micro-encapsulation technique is important in masking bitter tasting medicines through coating of the drug particles by a suitable polymer; the drug has no contact with the taste buds in the mouth when dosage forms are given orally to patients [11-17].For example, the unpleasant taste of diclofenac sodium was masked using a micro-encapsulation method without affecting the active ingredient's release rate. In addition, the results demonstrated that the extent of taste masking was influenced by the presence of additives such as cellulose and lactose within the core, as well as the plasticizer and the core size was found to have an effect on the parent drug's release rate [15]. However, effective methods of avoiding unpleasant perception for adults are problematic for pediatric formulations.

Guaifenesin, the glyceryl ether of guaiacol, is one of the most commonly used OTC drugs. It is a component of numerous cough and cold preparations available world-wide, termed as an expectorant. This medication is used to loosen mucus and phlegm and eventually clear the symptoms of congestion resulting from a cold or allergy [18-23]. Guaifenesin (**Figure 1**) was discovered in the 1500's, but its strong bitter taste made it unacceptable for pediatric and geriatric patients [20].

Since most of the above mentioned physical approaches were found to be limited, different strategies should be developed in order to overcome the unpleasant taste of guaifenesin.

Bitterness detection in the oral cavity of humans is facilitated by a family of ~25 bitter taste receptors (TAS2Rs) belonging to the G protein-coupled receptor (GPCR) super-family [24-26]. Despite low sequence similarity, TAS2Rs are classified as class A GPCRs [27]. The human sense of bitter taste is commonly categorized as a warning sense against the ingestion of toxic food components, although, in particular during later phases in life, moderate bitterness is well tolerated and even appreciated in the context of some food items and

beverages [28]. On the tongue, subsets of TAS2R genes are co-expressed in bitter taste receptor cells that represent a subpopulation of type II cells within taste buds [29,30].The functional screening of human TAS2Rs has so far resulted in the identification of bitter agonists for 21 of the 25 receptors Moreover, it became evident that TAS2Rs recognize a highly variable number of structurally diverse bitter compounds ranging from single or few compounds to dozens [31-34] . These differences in tuning breadths suggest that receptors may contribute disproportionally to human bitter sensing. Among the 3 most broadly tuned receptors, TAS2R10 [35], TAS2R14 [36], and TAS2R46 [37], the TAS2R14 exhibits to date the largest panel of identified bitter agonists, which frequently represent drugs with profound pharmacological activities [38].

The recent novel prodrug chemical approach invoked by Karaman's group involves the design of prodrugs for masking the bitter taste of pharmaceuticals based on intramolecular processes. In this approach no enzyme is needed to catalyze the reconversion of a prodrug to its corresponding parent drug. The rate of the drug release is controlled by the nature of the promoiety bound to the drug. The role of the latter is to block those functional groups located on the parent drug and responsible for the interactions between the drug and its bitter taste receptor/s [8, 9].This approach succeeded to design paracetamol prodrugs that mask the bitterness of the parent drug [39].Similarly, guaifenesin prodrugs may reduce or eliminate the parent drug's bitterness by altering its ability to interact with the cognate bitter taste receptor/s [39-42].

In this study, guaifenesin prodrugs consisting of mono- and di-esters were designed and synthesized aiming to provide non-bitter entities which upon exposure to the stomach medium undergo cleavage to provide the parent drug, guaifenesin and a non-toxic linker.

2. Material and methods:

2.1 Computational analyses

QM studies - The Becke three-parameter, hybrid functional combined with the Lee, Yang, and Parr correlation functional, denoted B3LYP, were employed in the calculations using density functional theory (DFT). All calculations were carried out based on the restricted Hartree-Fock method using the quantum chemical package Gaussian-2009 [43].

The starting geometries of all calculated molecules were obtained using the Argus Lab program [44] and were initially optimized at AM1 and HF/6-31G (d,p) level of theory, followed by optimization by DFT at the B3LYP/6-31G(d,p) level. The search for the global minimum structure in the guaifenesin derivatives studied was accomplished by 36 rotations around the ester O-C(O) bond in increments of 10°, and calculation of the energies of the resulting conformers. An energy minimum (a stable compound or a reactive intermediate) has no negative vibrational force constant. Transition states were located first by the normal reaction coordinate method [45] where the enthalpy changes were monitored by stepwise changing the interatomic distance between two specific atoms. The geometry at the highest point on the energy profile was re-optimized by using the energy gradient method at the B3LYP/6-31G (d,p) level of theory [46]. The "reaction coordinate method" was used to calculate the activation energy in the designed mono- and di-ester prodrugs. In this method, one bond length is constrained for the appropriate degree of freedom while all other variables are freely optimized [47]. The activation energy values for the prodrug interconversion to guaifenesin were calculated from the difference in energies of the global minimum structures (GM) and the derived transition states. Verification of the desired reactants and products was accomplished using the "intrinsic coordinate method" [48]. The transition state structures were verified by their only one negative frequency. Full optimization of the transition states was accomplished after removing any constrains imposed while executing the energy profile. The activation energies obtained from DFT at B3LYP/6-31G (d,p) level of theory for **GDEProD1**-**3**and **GMEProD1**-**3**(**Figure 1**) were calculated with and without the inclusion of solvent (water and ether). The calculations with the incorporation of a solvent were performed using the integral equation formalism model of the Polarizable Continuum Model (PCM) [49, 50].

2.2 Chemistry

The chemicals used in the present study were procured from Sigma Aldrich (USA). IR spectra were obtained from a KBr matrix (4000–400 cm^{-1}) using a Thermo Scientific Nicolet iS10 FT-IR Spectrometer. The LC-MS system used was LC system coupled to a hybrid quadrupole ion trap (LTQ) - Fourier transforms ion cyclotron resonance (FT-ICR) mass spectrometer (Thermo fisher scientific Bremen, Germany). 1H nuclear magnetic resonance (NMR) experiments were performed with I 500 MHz NMR Varian Unity INOVA

spectrometer (University of Basilicata). Samples were injected into Agilent technologies 1200 series (Avondale, PA, USA) HPLC-PDA. The optimal HPLC British pharmacopoeia validated method (with some modification in the percentage of the mobile phase component) used for the analysis of guaifenesin [51]; Eclipse XDB-C18 (3 µm particle size, 4.6 mm × 150 mm) column (Phenomenex-USA), a mixture of water: acetonitrile (water pH adjusted to 5.5 using diluted phosphoric acid) (55:45 v/v) as a mobile phase, a flow rate of 0.5 mL/minute and a diode array 1200 HPLC system consisting of an HP-G1311A Quat pump, HP G1311A UV detection at a wavelength of 276 nm in University of Basilicata.

2.2.1Synthesis of the Prodrugs

*2.2.1.1 Guaifenesin mono-ester prodrugs,**GMEProD1-3*(**Scheme S1**)*:*

In a 250 mL round-bottom flask guaifenesin (5 mmol) was dissolved in tetrahydrofuran (THF) (50 mL), 0.125 g (5 mmol) of sodium hydride (60% suspended powder) was added, the resulting solution was stirred for 30 minutes then a THF solution of 5 mmol maleic anhydride, succinic anhydride or glutaric anhydride, was slowly added to the reaction mixture which then was stirred at room temperature for overnight. The reaction was monitored by Thin Layer Chromatography (TLC) which was performed on regular basis to check the reactions completion. 1N HCl (50 mL) was added while the round-bottom flask was placed in an ice bath. The aqueous layer was extracted with ether (90 mL) three times and the combined ether layer was dried over $MgSO_4$ anhydrous, filtered and evaporated to dryness, then the product was washed with hexane three times and dried.

2.2.1.2 Guaifenesin di-ester prodrugs, ***GDEProD1-3*** (**Scheme S2**)***:***

In a 250 mL RBF guaifenesin (5 mmol) was dissolved in tetrahydrofuran (THF) (50 mL), 0.250 g (10 mmol) of sodium hydride (60% suspended powder) was added, the resulting solution was stirred for 30 minutes then a THF solution of 10 mmol 2,3-dimethyl maleic anhydride, maleic anhydride or 2,2-dimethyl succinic anhydride, was slowly added to the reaction mixture which then was stirred at room temperature for overnight. The reaction was monitored by Thin Layer Chromatography (TLC) which was performed on regular basis to check the reactions completion. 1N HCl (50 mL) was added while the round-bottom flask

was placed in an ice bath. The aqueous layer was extracted with ether (90 mL) three times and the combined ether layer was dried over $MgSO_4$ anhydrous, filtered and evaporated to dryness, then the product was washed with hexane three times and dried (**Figure 1**).

2.2.2Characterization by H-NMR, FTIR and LC-MS:

Guaifenesin Drug: ^{1}H NMR ($CDCl_3$, δ ppm) δ 7.01 – 6.69 (m, 4H), 4.19 – 3.85 (m, 5H), 3.84 (s, 3H), 3.81 (s, 1H), 3.27 (s, 1H). FT-IR (KBr/ν_{max} cm^{-1}): (O-H) 3251cm^{-1}, (C=C of C_6H_5) 1596 cm^{-1}. ^{13}C NMR ($CDCl_3$, δ ppm) δ 149.50, 148.10, 121.59, 121.20, 114.30, 112.00, 71.30, 70.30, 63.80, 56.10 (**Figure S1**).

GMEProD1*:* Yield: 61.5%, Solid (decomposes before melting), ^{1}H NMR ($CDCl_3$, δ ppm): δ 6.88-7.15 (m, 4H), 6.31-6.14 (m, 2H), 4.27 (m, 1H), 4.03-4.23 (m, 2H), 3.98-4.10 (m, 2H), 3.86 (s, 3H). FT-IR (KBr/ν_{max} cm^{-1}): (O-H) 3063 cm^{-1}, (C=O) 1738 cm^{-1}, (CH=CH) 1634 cm^{-1}. *m/z:* 295.08(100%), 295.11(25%), 295.14(12%) (M-1) (**Figure S1**).

GMEProD2*:* Yield: 58%, Oily,^{1}H-NMR ($CDCl_3$, δ ppm) δ 6.88-7.12 (m, 4H), 4.48 (m, 1H), 4.09-4.31 (m, 2H), 4.03-4.24 (m, 2H), 3.84 (s, 3H), 2.97 (m, 4H). FT-IR: (KBr/ν_{max} cm^{-1}): (O-H) 3017 cm^{-1}, (C=O) 1738cm^{-1}, and *m/z:* 297.09 (100%), 298.10(13.9%) (M-1) (**Figure S1**).

GMEProD3*:* Yield: 58%, Oily,^{1}H NMR ($CDCl_3$, δ ppm) δ 6.87-7.09 (m, 4H), 4.50 (m,1H), 4.16-4.33 (m, 2H), 4.05-4.24 (m, 2H), 3.70 (s, 3H), 2.39-2.45 (m, 4H), 1.91-1.97 (m, 2H). FT-IR: (KBr/ν_{max} cm^{-1}): (O-H) 3066 cm^{-1}, (C=O) 1736 cm^{-1}, and *m/z* 311.113 (100%), 312.116(13.0%) (M-1) (**Figure S1**).

GDEProD1: Yield: 73%, Oily,^{1}H NMR ($CDCl_3$, δ ppm) δ 11.10 (s, 2H), 7.07 – 6.60 (m, 4H), 5.28 (p, J = 5.0 Hz, 1H), 4.48 (d, J = 5.0, 1.4 Hz, 2H), 4.21 (d, J = 5.0, 1.4 Hz, 2H), 3.84 (s, 3H), 2.02 – 1.69 (m, 12H). FT-IR: (KBr/ν_{max} cm^{-1}): (O-H) 3220 cm^{-1}, (C=O) 1720 cm^{-1}, (C=C) 1637. ^{13}C NMR ($CDCl_3$, δ ppm) δ 170.77 – 170.59 (m, 2C), 169.28 (2C), 149.50 (1C), 148.10 (1C), 138.82 (1C), 138.63 (1C), 138.34 (d, J = 3.0 Hz, 2C), 121.59 (1C), 121.20 (1C), 114.30 (1C), 112.00 (1C), 74.61 (1C), 69.16 (1C), 62.39 (1C), 56.10 (1C), 15.36 – 14.04 (m, 4C), and *m/z* 449.15 (100%), 450.16 (25.3%), 451.12 (5.1%) (M-1) (**Figure S1**).

GDEProD2: Yield: 80%, Oily,^{1}H NMR ($CDCl_3$, δ ppm) δ 13.06 (s, 2H), 7.10 – 6.56 (m, 4H), 6.33 – 5.38 (m, 4H), 5.38 – 5.06 (m, 1H), 4.34 (d, J = 5.0, 1.4 Hz, 4H), 3.84 (s, 3H). FT-IR:

(KBr/ν_{max} cm^{-1}): (O-H) 3224 cm^{-1}, (C=O) 1734 cm^{-1}, (C=C) 1644 cm^{-1}. ^{13}C NMR ($CDCl_3$, δ ppm) δ 167.03 (1C), 166.55 – 166.40 (m, 3C), 149.50 (1C), 148.10 (1C), 136.01 – 135.41 (m, 2C), 132.46 (1C), 131.01 (1C), 121.59 (1C), 121.20 (1C), 114.30 (1C), 112.00 (1C), 73.06 (1C), 69.16 (1C), 62.39 (1C), 56.10 (1C)and *m/z* 393.09 (100%), 394.07 (20.4%), 395.10 (2.2%) (M-1) (**Figure S1**).

GDEProD3: Yield: 90%, Oily,^{1}H NMR ($CDCl_3$, δ ppm) δ 12.00 (s, 2H), 7.00 – 6.70 (m, 4H), 5.28 (t, J = 5.0 Hz, 1H), 4.26 (d, J = 15.0, 5.0 Hz, 4H), 3.84 (s, 3H), 2.72 (d, J = 3.7, 1.9 Hz, 4H), 1.16 (s, 12H). FT-IR: (KBr/ν_{max} cm^{-1}): (O-H) 3212 cm^{-1}, (C=O) 1732 cm^{-1}. ^{13}C NMR ($CDCl_3$, δ ppm) δ 178.00 – 177.72 (m, 2C), 170.93 (1C), 170.42 (1C), 149.50 (1C), 148.10 (1C), 121.59 (1C), 121.20 (1C), 114.30 (1C), 112.00 (1C), 71.65 (1C), 69.16 (1C), 62.14 (1C), 56.10 (1C), 46.23 (1C), 45.61 (1C), 43.58 (2C), 25.14– 24.92 (m, 4C),and *m/z* 453.18 (100%), 454.17 (25.3%), 455.19 (5.1%) (M-1) (**Figure S1**).

2.2.3Kinetic Methods

After the preparation of the calibration curves for guaifenesin and all the prodrugs, a sample from each prodrug was prepared in 1N HCl, buffer pH 3.3, buffer pH 5.5 or buffer pH 7.4, and all samples were injected into HPLC-PDA using the method mentioned above.

2.2.3.1Hydrolysis of guaifenesin prodrugs:

Guaifenesin prodrugs hydrolysis rates were studied at 37 ^{0}C in buffer solutions at different pHs (1N HCl (pH 0.2), 0.1N HCl (pH 1.2), pH 3.3, pH 5.5 and pH 7.4). Samples from the reaction mixtures were analyzed directly by HPLC; the decreased area percentage of the prodrug peaks and the increased area of guaifenesin peak were monitored during the reaction progress, then area under the peaks of guaifenesin and its prodrugs vs. time were plotted.

2.3 Functional calcium imaging experiments

Functional heterologous expression of TAS2R14 was done as previously reported [52]. Briefly, TAS2R14 cDNA flanked by a 5' sst3-tag and a 3' hsv-tag in the vector pcDNA5FRT [36] was transiently transfected into HEK 293T-Gα16gust44 cells. After ~24 h cells were loaded with Fluo-4 AM in the presence of 2.5 mM probenecid, then washed several times with C1-buffer and placed in a fluorometric imaging plate reader ($FLIPR^{tetra}$, Molecular Devices). Test stimuli were automatically applied and changes in fluorescence were

monitored. Cells transfected with an empty expression vector were used as a negative control and directly compared to receptor transfected cells. Two independent experiments performed in triplicates were used for the calculations.

2.4 Molecular modeling

TAS2R14 receptor model generated in Karaman et al. and refined in Di Pizio et al [52.] was used to investigate the binding modes of guaifenesin, by using the Induced-Fit docking protocol (Glide version 7.5; Prime version 4.8, Schrödinger, LLC, New York, NY, 2017) [53,54]. Side chains of residues within 5.0 Å of the ligand were refined. The docking was performed with the Standard Precision (SP) mode of Glide. The binding poses of the prodrugs were obtained aligning the ligand structures to the guaifenesin in its docking pose with Phase Shape Screening (Phase, version 5.1, Schrödinger, LLC, New York, NY, 2017). Therefore binding energies of the ligands with receptor were estimated by employing the Multi Ligand Bimolecular Association with Energetics (MBAE) [55], using EMBRACE minimization of Macro Model (Schrödinger, LLC, New York, NY, 2017). All complexes have been minimized to a derivative convergence of 0.05 kJ/mol Å using the Polak-Ribiere Conjugate Gradient (PRCG) minimization algorithm, the OPLS3 force field. A shell of 5 Å around the ligand was set to be free to move, another shell of 3 Å minimized applying a force constant of 200 kJ/mol Å2.

Embrace minimization was performed by opting energy difference mode. The calculation was performed first on the receptor, then on the ligand, and finally on the complex. The energy difference is then calculated using the equation:

$$\Delta E \text{ (ligand binding energy)} = E_{complex} - E_{ligand} - E_{protein}$$

Since all compounds were synthesized and tested in racemic mixture, we have submitted all stereoisomers to the simulations and used the stereoisomers the showed better performance for the analysis.

Transmembrane (TM) residues are identified by a superscript number system according to the Ballesteros-Weinstein numbering method [56].

3. Result and discussion:

We have successfully obtained six guaifenesin prodrugs with five different linkers, **GDEProD1**, **GDEProD2**, **GDEProD3**,**GMEProD1**, **GMEProD2**, **GMEProD3** (**Schemes S1**and **S2**). These prodrugs were characterized by FT-IR, 1H-NMR and LC-MS techniques as shown in **Figures S1 (A,B and C)**.

The kinetics of the acid-catalyzed hydrolysis for guaifenesin prodrugs were carried out in aqueous buffers in a manner similar to that done by Bruice's group on methyl phenyl acetals of formaldehyde [57]. The kinetic study aimed to investigate whether guaifenesin prodrugs are hydrolyzed to release the parent drug, guaifenesin in aqueous medium and to what extent. Acid-catalyzed hydrolysis of the synthesized guaifenesin prodrugs was studied in four different aqueous media: HCl and buffers pH 3, pH 5 and pH 7.4 and the hydrolysis reaction was monitored by HPLC (**Scheme S3**). The appearance of the parent drug's peak and disappearance of the prodrug's peak were monitored to determine the rates of the interconversion of each of the six prodrugs and the results are summarized in **Figures S2 (A,B and C)**.

For guaifenesin prodrugs, at constant temperature (37°C) and 1N HCl (pH 0.2), the reaction displayed strict first-order kinetics for **GDEProD1**, **GDEProD3** and **GMEProD1** as the k_{obs} was fairly constant and a straight line was obtained by plotting ln concentration of the guaifenesin prodrugs versus time in hours, and zero order for the other prodrugs according to the best linearity of concentration versus time plotting as shown in **Tables S1** and**S2.** The rate constant (k_{obs}) for guaifenesin prodrugs in 1N HCl was calculated from the linear regression equation correlating the concentration of the prodrug versus time according to the integral rate law equation of the zero order law (eq. 1), and the linear regression equation correlating the concentration of the prodrug versus time according to the integral rate law equation of the first order (eq. 2) (**Tables S1** and**S2**). The rate constant (k_{obs}) obtained from the kinetics for **GDEProD1**, **GDEProD3** and **GMEProD1** was found to be 5.2 x 10^{-3}, 0.02 x 10^{-3}, 1.6 x 10^{-3}mol x L^{-1} x h^{-1}, and 12.2 x 10^{-3}, 11.75 x 10^{-3}, 11.14 x 10^{-3}mol x L^{-1} x $h^{-1}$for **GDEProD2**, **GMEProD2**, and **GMEProD3**, respectively. The complete hydrolysis of the guaifenesin prodrugs to their parent drug, guaifenesin in 1N HCl required 1.67, 5.33, 11.00, 12.00, 13.5, and 96 hours, respectively. Whereas, in 0.1 N HCl the hydrolysis rate constant of **GEDProD1** was 14.01 x 10^{-3}mol x L^{-1} x h^{-1}as zero order reaction, and required 6 hours for complete hydrolysis to guaifenesin, as shown in **Figure S2 (D).**

Using the equations derived from the integral zero and first law (eqs. 3 and 4) and the experimental rate conversion value we have calculated the $t_{1/2}$ values (the time needed for the conversion of 50% of the reactants to products) for the conversion of **GDEProD1-3** and **GMEProD1-3** to their parent drug. The t½ values for **GDEProD1-3** and **GMEProD1-3** were 0.3, 4.81, 73.13, 1.45, 7.06 and 7.16 h in 1N HCl, and 3.81 h for **GDEProD1** in 0.1N HCl, respectively**.**

$$[\mathbf{A}] = [\mathbf{A}^\circ] - \mathbf{k} * \mathbf{t} \ldots\ldots \quad \mathbf{eq.1} \qquad \mathbf{ln}[\mathbf{A}] = \mathbf{ln}[\mathbf{A}^\circ] - \mathbf{kt} \ldots\ldots \mathbf{eq.2}$$

$$\mathbf{t\,(half)} = [\mathbf{A}^\circ]/\mathbf{2k}] \ldots\ldots \mathbf{eq.3} \qquad \mathbf{t(half)} = \frac{\mathbf{ln2}}{\mathbf{k}} \ldots\ldots \quad \mathbf{eq.4}$$

Where $[A]=[A^0]/2$, $[A_t]$ is the concentration during time and [A] is the initial concentration.

3.1 Computational analysis

Continuing the strategy for exploring enzyme models in the design of novel prodrugs, Bruice's enzyme model (hydrolysis of di-carboxylic semi-esters) was employed in the design of guaifenesin prodrugs (**Schemes S1**and **S2**) which have the potential to be more bioavailable and to elicit less bitter sensation. Furthermore, it is planned that their intra-conversion rate to guaifenesin will be programmed according to the nature of the prodrug linker [57,58].

The calculations at B3LYP/6-31G (d,p) level for the ring-closing reactions of di-carboxylic semi-esters, **GMEProD1-3** and **GDEProD1-3** (**Schemes S1** and**S2**), were directed toward elucidation of the transition and ground state structures (reactants, intermediates and products). Calculations for all ground states, intermediates, transition states and products were run in water (dielectric constant of 78.39) and in the gas phase. It is expected that the stability of the ground and transition states will be different in solvent having low dielectric constant, such as the gas phase and solvent with high dielectric constant, such as water.

The orientation of the carboxylate anion to the ester carboxyl moiety is very important and affects the mode and rate of the ring-closing reaction. The distance (d_{GM}) between the nucleophile (O1) and the electrophile (C6) is shorter in the di-carboxylic semi-ester exists in

the *syn* (condensed) conformation than the *anti* (extended) conformation (**Scheme S3**). This is the reason that the cyclization in *syn* conformation is more efficient than in the *anti* (extended) conformation.

In addition, the activation energy of the prodrug to drug intraconversion is largely affected by the strain energy of the prodrug conformation. If the ground state is strained, the free energy of the reactant will be higher than the less strained conformation, so it is predicted that the activation energy of the strained reactant conformers will be less than that of the less-strained conformers. We were concerned with the identification of the most stable conformation (Global Minimum, GM) for each of dicaboxylic semi-esters **GMEProD1-3** and **GDEProD1-3**.

The DFT calculations of the starting geometries in **GMEProD1-3** and **GDEProD1-3** demonstrated that the global minimum structures (GM) for all prodrugs exist in the *syn* (condensed) conformation, as shown in **Figure S3 (A).**

3.1.1Optimized Geometries for the Entities Involved in the Ring-Closing Reactions of GMEProD1-3 and GDEProD1-3

3.1.1.1Reactants (GM)

The global minimum structures for **GMEProD1-3** and **GDEProD1-3** are illustrated in **Figure S3 (A).** The d_{GM} values for **GDEProD1-3** were 3.147Å, 3.254 Å and 4.115 Å, respectively, and for **GMEProD2-3** were 3.468 Å 3.497 Å, respectively, where the GM for **GDEProD1** was with the shortest distance and for the GM of GDEProD3 with the longest distances.

3.1.1.2Transition State Geometries (TS)

The calculated DFT transition state structures for the cyclization reactions of **GMEProD1-3** and **GDEProD1-3** are shown in **Figure S3 (B)**. Examination of the optimized TS structures indicates that all of them resemble that of the corresponding tetrahedral intermediates. Furthermore, the calculated O-C distances, O1-C6, O8-C6 and O1-C2 (**SchemeS4**) are significantly different. The distance range for O1-C6 in the TS of **GMEProD1-3** and TS of **GDEProD1-3** was 1.378 Å – 1.449 Å, for O8-C6 was 1.792 Å – 1.803 Å and for O1-C2 was 1.371 Å - 1.413 Å.

3.1.2Reaction Mechanism Investigation

The DFT calculations at B3LYP 6-31G (d,p) level were performed for calculating the kinetic parameters for all entities involved in the reaction shown in **Scheme S5**. The mechanism involves two steps; the first is the approach of the anionic carboxylate oxygen (O1) toward the carboxylic carbon (C6) to give a tetrahedral intermediate and the second is the dissociation of the tetrahedral intermediate to yield a cyclic anhydride and guaifenesin-enolate anion. The 'reaction coordinate' calculations for both steps revealed the following: (a) no transition state structures were found for the approach processes in **GMEProD1-3** and **GDEProD1-3**. (b) The 'reaction coordinates' and frequency calculations for the intermediate dissociation route in **GMEProD1-3** and **GDEProD1-3** demonstrated the presence of a transition state. Further, monitoring the dissociation processes revealed that upon increasing the distance between O8 and C6, opening of the cyclic ring was observed in **GMEProD1-3** and **GDEProD1-3**. However, the cycle opening magnitude (the distances O1-C6, O8-C6 and O1-C2) was found to be dependent on the transition state nature.

Using the calculated B3LYP 6-31G (d,p) values for the enthalpy (H) and entropy (S) of the global minimum (GM) and the transition state (TS) structures for the ring-closing reactions of di-carboxylic esters **GMEProD1-3** and **GDEProD1-3**, (**Table S3**), the activation energy values for the rate-limiting step (ΔG) were calculated in the gas phase as well as in a dielectric constant of 78.39 (water). The calculated activation energy values in both media, ΔG_{GP} and ΔG_{H2O}, respectively, are summarized in **Table S4**. Inspection of the ΔG values shown in Table S4 demonstrated that the cyclization rate is largely affected by the nature (structural features) of the reactant.

To examine whether the discrepancy in the rates for the reactions of **GMEProD2-3** and **GDEProD1-3** stems from proximity orientation or is due to steric effects (strain energy) we calculated using molecular modeling (MM2) method the strain energy values (E_s) for the reactants and intermediates in systems **GMEProD2-3** and **GDEProD1-3**. The calculated E_s values for the reactions of **GMEProD2-3** and **GDEProD1-3** were examined for the correlation with the experimental $t_{1/2}$ values and the correlation results are depicted in equations 5 and 6 and illustrated graphically in **Figure S3(C, I)**. Eq. 5 and **Figure S3 (C, I)** demonstrate a good correlation between the experimental $t_{1/2}$ and the MM2-calculated strain energy values (E_s) with a correlation coefficient (R) of 0.94.

It should be noted, that attempts to correlate the distance O1-C6 (d_{GM}) with $t_{1/2}$ gave random correlation. For example, the calculated O1-C6 distances for **GDEProD1** and **GMEProD2**were similar (3.147 Å- 3.254 Å), whereas the calculated Es and $t_{1/2}$ experimental values differ significantly ($t_{1/2}$; 0.3 h and 1.45h, Es: 22.2 and 10.61, respectively). These results suggest that the driving force for the enhancement in the ring-closing reaction is driven by strain effects in contrast to that suggested by Bruice et al. [57-58]. In order to further support this conclusion, the B3LYP 6-31G (d,p) for **GMEProD2-3** and **GDEProD1-3** activation energy values in water (ΔG_{H2O}) were examined for correlations with both $t_{1/2}$ and Es and the results are shown in equations 7-8 and represented graphically in **Figure. S3(C**, **II,III**).

Again, the correlation results of the calculated ΔG_{H2O} with the experimental half-lives ($t_{1/2}$) revealed to the same conclusions; the driving force for acceleration is due to strain effects and not to proximity orientation.

$Es = 0.6797\ t_{1/2} + 16.271$ (R=0.9)……..(eq. 5)

ΔG_{H2O} (B3LYP/6-31G) $= 1.4042\ t_{1/2} + 3.2675$ (R=0.9)…….(eq. 6)

ΔG (B3LYP/6-31G) $= 1.3469\ E_s + 19.351$ (R=0.9)……(eq. 7)

$\ln [A_t] = \ln [A_0] - K.t$, ($[A_t] = [A_0]/2$ (for $t_{1/2}$ calculation)……(eq. 8)

The half-lives, $t_{1/2}$ for Processes GDEProD1-3 and GMEProD1-3

The experimental half-lives for the intramolecular ring-closing reactions of **GDEProD1-3** and **GMEProD1-3** were obtained from the integral first order law (eq. 8).

Since an excellent correlation was obtained between the activation free energy values (ΔG_{H2O}) for **GDEProD1-3** and **GMEProD1-3** and the strain energy values (eq. 7), the calculated values of E_s for **GDEProD1-3** and **GMEProD1-3** were used in eq. 5 to calculate the $t_{1/2}$ in equation 7 as shown in eq 7, half-life= [(ΔG_{H2O} (B3LYP/6-31G))/1.3469 – 16.271]/0.6797. The calculated half-lives for **GDEProD1-3** and **GMEProD1-3** obtained were: 5.69, 19.10, 3.78, 10.826, 26.513 and 29.610 h, respectively. Examination of the calculated half-lives demonstrates that **GDEProD1** and **GMEProD1** are the most efficient

processes among all the systems investigated, whereas the least efficient are **GMEProD2** and **GMEProD3**.

Using the integral rate law first order equation (eq. 8) the calculated rate conversion for **GDEProD1** and**GDEProD3** and **GMEProD1-3** obtained were: 0.1217, 0.0640, 0.0362, 0.0261, and 0.0234 h^{-1}, respectively.

For obtaining credibility to our calculation results, we introduce our computation rational for calculating the (ΔG) values for **GDEProD1-3** and **GMEProD1-3** based on the DFT calculated enthalpy of activation (ΔH) and entropy of activation for **GDEProD1-3** and **GMEProD1-3**, experimental free activation energies ($Exp_{\Delta G}$) were calculated using equation 9, which describes the ΔG parameter as a function of the rate conversion values.

$\Delta G = -RT \ln K$ ……..(Eq. 9)

Where R is the Ideal Gas constant (1.9872036×10^{-3} kcal/mol K) and T is the temperature in Kelvin.

The $Exp_{\Delta G}$ for **GDEProD1-3** were 3.114, 4.345, 6.406 kcal/mol x Kelvin, and for **GMEProD1-3** were, 3.811, 4.500 and 4.524 kcal/mol x Kelvin, respectively.

In addition, for further support to the credibility of our DFT calculations, the calculated free activation energies in water (ΔG) were correlated with the experimental free activation energies ($Exp_{\Delta G}$). Strong correlation was obtained with R value of 0.92 (**Figure S3 (D)**). **Figure S3(D)**indicates that although the calculated and experimental ΔG values are comparable, their absolute values slightly differ. This might be due to the fact that the experimental ΔG values for **GDEProD1-3** and **GMEProD1-3** were measured in the presence of aqueous acid, whereas the DFT calculations were run in plain water. The dielectric constant value for a mixture of acid/water is expected to be different from pure water (78.39) and hence the discrepancy in the calculated and experimental ΔG values.

3.2 TAS2R14 activation

To investigate if the derivatization of guaifenesin may enable the creation of TAS2R14 non-agonists which can be bioactivated, we challenged HEK 293T-Gα16gust44 cells transiently transfected with TAS2R14 cDNA with different concentrations of derivatives and the parental substance guaifenesin and monitored calcium responses (**FigureS4**)Whereas guaifenesin, guaifenesin maleate, guaifenesin glutarate, and guaifenesin

dimaleate elicited cellular responses, the two succinate derivatives guaifenesin succinate and guaifenesin disuccinate failed to activate TAS2R14 expressing cells at all concentrations. Hence, derivatization of guaifenesin with succinate would prevent bitterness mediated by TAS2R14, whereas other modifications would not eradicate TAS2R14 activation or even, in case of guaifenesin glutarate, result in increased responses. As the masking of the guaifenesin moiety should not be too different between e.g. the agonist guaifenesin dimaleate and the non-agonist guaifenesin disuccinate, the succinate side-chains may adopt a conformation that prevents receptor activation.

3.3 Structural analysis

We have investigated the conformation assumed by guaifenesin in the receptor's binding pocket [27], by Induced Fit Docking simulations [53,54]. Guaifenesin establishes π-π interactions with Phe2476.55, hydrophobic interactions with Phe1865.46, and H-bonds with Asn933.36 and Ser2657.38 side chains of TAS2R14 (**FigureS5**). We have aligned prodrugs to guaifenesin in the predicted binding conformation in order to maintain the binding pose and the main interactions, i.e. π-π interactions with Phe2476.55 and H-bond with Asn933.36. This allowed us to understand how the chemical modifications of synthesized prodrugs affect TAS2R14 activity in comparison to the bitter guaifenesin. Guaifenesin glutarate (GMEProD3) can establish π-π interactions with Phe1865.46 and points the glutarate ester towards TMs 6 and 7, establishing H-bonds with Ser2657.38 and Ser2466.54. Moreover, the ligand conformation is stabilized by an intramolecular H-bond between the carboxyl and the hydroxyl groups (**FigureS5**). Guaifenesin maleate (GMEProD1) binds to TAS2R14 in a very similar mode establishing the same interactions observed for the glutarate derivative (**FigureS5**). The additional maleate moiety of GDEProD2 points to the extracellular region and forms H-bonds with Asn157, Ser167 and Ser169 of the ECL2 (**FigureS5**). This breaks the intramolecular H-bond and causes a slight modification of the conformation of the carboxyl group that interacts with Ser2657.38 and Ser2466.54, and a different orientation of the aromatic ring that cannot establish the π-π interactions with Phe1865.46, as observed for GMEProD3 and GMEProD1. Guaifenesin succinate and disuccinate show binding modes similar to guifenesin maleate and dimaleate but the saturated esters increase the volume of the molecules that clash with Phe2476.55, whose position is restrained because of the π-π interactions with the aromatic ring (**FigureS5**).

4. Summary and Conclusion

Taken together we have shown that prodrugs of guaifenesin can be rationally designed. Using multiple derivatization strategies, a set of prodrugs has been established showing sufficient stability at neutral pH-values and rapid release under low pH-conditions as observed during stomach passage. Using bitter taste receptor assays, we confirmed that the most promiscuous human bitter taste receptor responsible for the bitter perception of many drugs, the TAS2R14, is not activated by some of the prodrugs. We conclude that our approach could be used to design better drugs showing reduced bitterness in particular for pediatric formulations.

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6. Conflict of Interest

The authors declare no conflicts of interest.

7. Figure 1 legend:

Figure 1:. Chemical structures of: 1. guaifenesin, 2. GMEProD1, 3. GMEProD2, 4. GMEProD3, 5. GDEProD1, 6. GDEProD2, and 7. GDEProD3.

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