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ORIGINAL PAPER

Effect of dehydroleucodine on intestinal transit: structural basis of the interaction with the α_2-adrenergic receptor

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Abstract The activity of dehydroleucodine, a sesquiterpene lactone obtained from Artemisia douglasiana, was studied in mice small intestinal transit. Its mechanism was evaluated in the presence of several adrenergic and cholinergic antagonist drugs and one opioid antagonist. Docking of dehydroleucodine into the homology model of the α2-adrenergic receptor allowed us to analyze the structural basis of their interactions. The experiments showed that dehydroleucodine delayed intestinal transit. The docking of dehydroleucodine showed a unique binding site, equivalent to the binding site of carozolol in the β-adrenergic receptor. The results suggested that dehydroleucodine produced an inhibitory effect on intestinal transit. Its action could be mediated, at least in part, through the α2-adrenergic receptor.

Keywords Dehydroleucodine · Small intestinal transit · Docking · α2-adrenergic receptor

Introduction

There are two basic types of movements that can occur in the gastrointestinal tract: *mixing movements*, which keep the intestinal contents thoroughly mixed at all times, and *propulsive movements*, which cause food to move forward along the tract at an appropriate rate for digestion and absorption. The movement of the small intestine, as elsewhere in the gastrointestinal tract, has an intrinsic nervous system composed of myenteric and submucosal plexus. In general, stimulation of the myenteric plexus increases tonic contraction, increases the intensity of the rhythmic contraction and increases velocity of the conduction. The excitatory fibers are cholinergic innervations that secrete acetylcholine as an excitatory transmitter that activates the muscarinic receptor.

The gastrointestinal tract has also adrenergic innervations. In general, these systems inhibit activity in the gastrointestinal tract. The sympathetic fibers secrete noradrenaline, a neurotransmitter that activates α- and β-receptors. When the sympathetic nerves are stimulated they promote an effect that is essentially opposite to those of the cholinergic system. On the other hand, some mesenteric plexus fibers are inhibitory rather than excitatory, and they are believed to be purinergic.

In Argentina, *Artemisia douglasiana* Besser is used in folk medicine and known under the common name of "matico." The popular use of matico infusions is to treat peptic ulcers and gastrointestinal disorders (Ariza Espinar and Bonzani 1992).

Giordano et al. (1990) isolated a sesquiterpene lactone, known as dehydroleucodine (DhL), from the aerial parts of *Artemisia douglasiana*. This lactone was studied in several preclinical assays; Piezzi et al. (1995) and María et al. (1998) reported that DhL shows gastric and duodenal cytoprotective activity plus anti-*Helicobacter pylori* activity (Vega et al. 2009).

In our previous work we have demonstrated that DhL prevents damage and inflammation, producing a significant

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decreases in diarrhea (Wendel et al. 1999; Wendel et al. 2007).

The objective of the present study is to report the effect of DhL upon small intestinal transit in mice and to investigate its action in the presence of adrenergic, cholinergic and opioids drugs that have well known mechanisms. We probe the structural basis of the interaction with the α_2-adrenergic receptor.

Materials and methods

Pharmacological assay

Drugs

DhL was extracted as previously described (Giordano et al. 1990). Briefly, the air-dried material was soaked in $CHCl_3$ at room temperature, evaporated in a vacuum and dissolved in EtOH. After addition of 4% aqueous lead tetra-acetate solution, the cloudy solution was filtered through a Celite pad, and the filtrate was concentrated in vacuum. The residue was extracted with $CHCl_3$, and the obtained solution was concentrated in vacuum. The final residue was chromatographed in a medium-pressure chromatography system using 1:9 AcOEt/hexane as eluent. DhL (100% purity) was identified by ^{1}H and ^{13}CNMR, MS and mp (Fig. 1).

Phentolamine (α_1, α_2-adrenergic receptor antagonist), prazocin (α_1-adrenergic receptor antagonist), yohimbine (α_2-adrenergic receptor antagonist), propranolol (β-adrenergic receptor antagonist), atropine (cholinergic receptor antagonist) and naloxone (opioid receptor antagonist) were purchased from Sigma USA.

Animals

Rockland mice (20–25 g) were used. They were housed in standard environmental conditions and fed with rodent diet and water ad libitum. All experiments were in compliance with the ANMAT (Administración Nacional de Medicamentos, Alimentos y Tecnología Médica) no. 6344/96 for animal care guidelines.

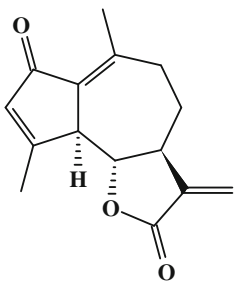

Fig. 1 Structure of dehydroleucodine

Effect of small intestinal transit in mice

The effect of DhL on small intestinal transit in mice was tested using the charcoal method (Di Carlo et al. 1993). Mice were pretreated orally with DhL at doses of 40, 60 and 80 mg/kg. The charcoal meal (a suspension containing 10% charcoal in 5% arabic gum; 0.1 ml of distilled water/10 g animal body) was administered 60 min after the administration of DhL. Mice were killed by cervical dislocation after 20 min, and the small intestine was rapidly removed and laid out on white filter paper for inspection and measurement of distances traversed by the charcoal. The length traversed by the charcoal marker was calculated as a percentage of the intestine length. To investigate the mechanism of action of DhL, different drugs acting with well-known mechanisms such as phentolamine 1 mg/kg, prazosin 1 mg/kg, yohimbine 1 mg/kg, propranolol 2.5 mg/kg, atropine 0.25 mg/kg and naloxone 10 mg/kg were used. These drugs were dissolved in 0.9% NaCl (saline) and given subcutaneously 10 min before DhL administration.

Statistical analysis

Data were expressed as mean ± SEM and analyzed by Student's *t* test for paired data.

Homology modeling

The three-dimensional model of the α_2-adrenergic receptor (A2AR) was constructed with MODELLER 9.7 (Sali and Blundell 1993), using as template the high-resolution crystallographic structure of the human β_2-adrenergic G protein-coupled receptor (HAGPCR) (PDB dataset 2RH1), which has 35% sequence identity and 55% similarity. BLAST (Altschul et al. 1997) was used to search for the templates and to do the alignment. The quality of the model was assessed by PROCHECK (Laskowski et al. 1993).

Docking of DhL and quercetin

The key results in a docking run are the docked structures found at the end of each run, the energies of these docked structures and their similarities to each other. The similarity of docked structures is measured by computing the root-mean-square deviation (rmsd) between the coordinates of the atoms. A docking experiment usually has several solutions. The reliability of a docking result depends on the similarity of its final docked conformations. One way to measure the reliability of a result is to compare the rmsd of the lowest energy conformations and their rmsd to one another, to group them into families of similar conformations or "clusters." The process involves ordering all the

conformations by docked energy, from lowest to highest. The lowest energy conformation is used as the seed for the first cluster. Next, the second conformation is compared to the first. If it is within the rmsd tolerance, it is added to the first cluster. If not, it becomes the first of a second cluster.

The docking of DhL and quercetin was done using AUTODOCK 4 (Scripps Research Institute. La Jolla, CA) (Morris et al. 1998; Huey et al. 2007; Sanner 1999). All water molecules were removed and nonpolar hydrogens were added using UCSF CHIMERA (Pettersen et al. 2004). Atomic partial charges for the receptor and the ligands were computed by the Gasteiger method (Gasteiger and Marsili 1980). The atomic interaction energy grids were calculated using probes corresponding to each atom type found in the substrate. They were tested every 0.375 Å in a 60-Å cubic box centered in approximately the putative active site of A2AR. The docking of the compound consisted of two stages or runs. In the first run the compound was placed in the center of the box. For the second stage, the global minimum structure, the low-energy structures of significant clusters were subjected to redocking under the same conditions and to cluster analysis using a 2.0-Å RMSD. Genetic algorithms with Local Search or Lamarckian with 250,000 evaluations were used for the dockings.

Results

Pharmacological assay

DhL caused a significant delay of normal intestinal transit at doses of 60 and 80 mg/kg. Phentolamine and yohimbine reversed the inhibitory effect of 80 mg/kg of DhL. The effect of DhL was not influenced by prazosin, propranolol, atropine and naloxone. The antagonist drugs did not influence *per se* small intestinal transit (Fig. 2).

α_2-Adrenergic receptor

The constructed homology model of the A2AR included 265 amino acids. N-terminus residues 1–50, residues 239–371 and C-terminus residues 449–458 were not modeled as they were disordered in the template. The fold was composed of seven transmembrane helices forming a helical bundle (Fig. 3).

The residues that form the helices are as follows: helix **I**, Val1 to Thr 28; helix **II**, Ala 34 to Met 64; helix **III**, Gln 71 to Thr 104; helix **IV**, Pro 115 to Val 138; helix **V**, Thr 158 to Lys 188; helix **VI**, Arg 189 to ILe 220; helix **VII**, Pro 228 to Val 252. The residues making up the intracellular loops (**ICLs**) and the extracellular loops (**ECLs**) are: **ICL1**, Ser 29 to Arg 33; **ECL1**, Ala 65 to Gly 70; **ICL2**,

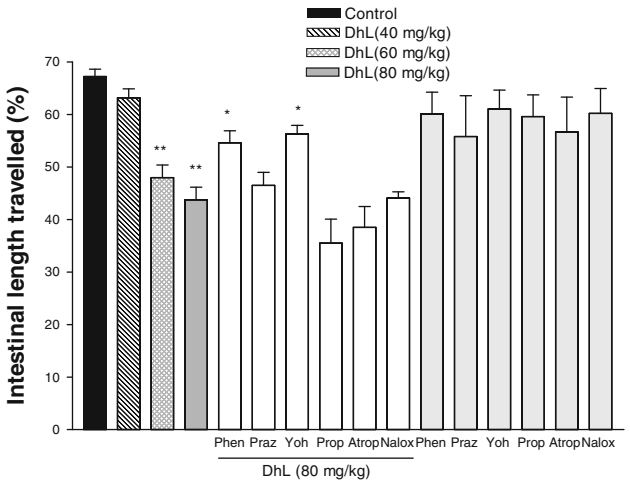

Fig. 2 Effect of phentolamine, prazosin, yohimbine, propranolol, atropine and naloxone on the inhibiting activity of DhL on small intestinal transit. Results were analyzed by Student's *t* test; $^{**}P < 0.001$ versus vehicle; $^{*}P < 0.01$ versus DhL 80 mg/kg

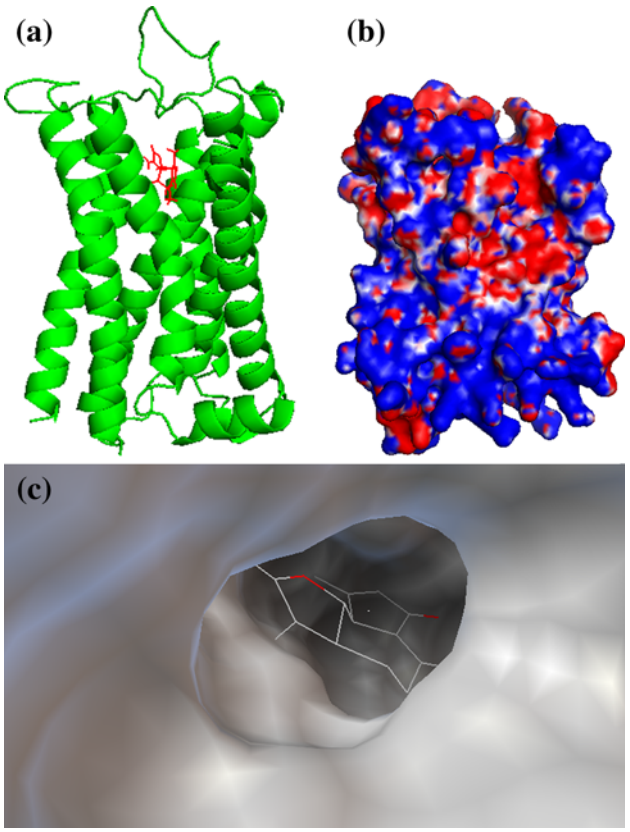

Fig. 3 **a** Overall fold of the α_2-adrenergic receptor with DhL bound in active site. PYMOL (DeLano 2002) was used in the preparation of all Figures. **b** Surface representation of the α_2-adrenergic receptor colored by calculed charge from *red* −1 to *blue* +1, using a dielectric constant of 78. Poisson-Boltzman electrostatic was calculed using APBS (Baker et al. 2001) as implemented in AUTODOCK. **c** Surface representation of the active site of the α_2-adrenergic receptor with bound DhL

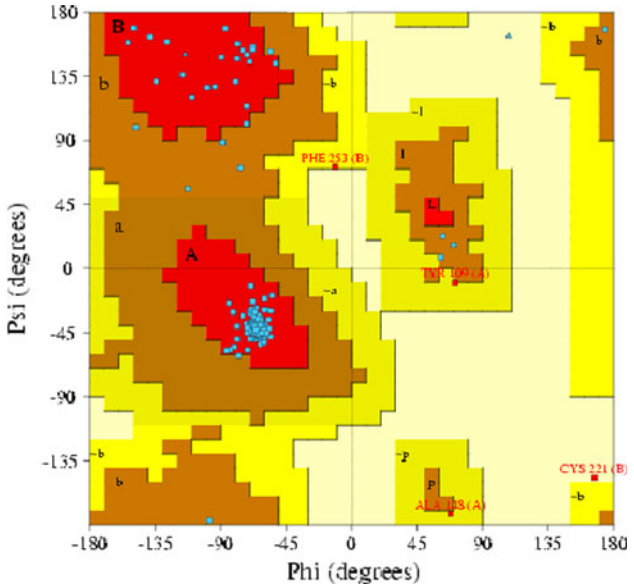

Fig. 4 Ramachandran plot of the receptor homology model

Gln 105 to Thr 114; **ECL2**, Leu 137 to Glu 157; **ICL3**, missing in the template; and **ECL3**, Cys 221 to Pro 228. The disulfide bond, Cys 74-Cys 152, that links helix **III** with **ECL2**, is conserved. The main topology differences between the template and the α_2-adrenergic receptor are the position of **ECL2**. This is due, perhaps, to an intraloop disulfide bond that is present in the template but missing in the α_2-adrenergic receptor. The Ramachandran plot of the receptor can be seen in Fig. 4.

Binding site

The extracellular loops and the N termini of GPCRs (G protein coupled receptors) together with the extracellular halves of the transmembrane helices are thought to define the ligand-binding site of each receptor (Angers et al. 2000). Small molecule ligands, like DhL, are believed to bind much deeper within the space generated by the transmembrane helices, whereas larger ligands such as peptides bind closer to the membrane surface near the extarcelular loops (Ji et al. 1998; Gether 2000). In the human β_2-adrenergic GPCR, the deep binding site defined by the binding of carozolol (Cherezov et al. 2007) is formed by the following residues making hydrophobic and aromatic interactions within a 4 Å radius: Trp 109 (77), Val 114 (82), Val 117 (Cys 85), Thr 118 (86), Phe 193 (Leu 154), Tyr 199 (160), Ser 207 (168), Trp 286 (208), Phe 289 (211), Phe 290 (212) and Tyr 308 (Phe 232). The salt bridge and hydrogen bond contacts are held by Asp 113 (81), Asn 312 (Phe 236) and Tyr 316 (240). The numbers within brackets are the corresponding residue number in A2AR. Although the overall sequence identity between HAGPCR and A2AR is about 35%, the binding site is highly conserved. The most important difference in the binding site is caused by the mutation of Phe 193 into Leu 154, which produced a widening of the binding site.

Docking of DhL and quercetin

The results of DhL docking showed that the lowest energy conformations were grouped into a single cluster at a binding energy −7.38. Therefore, the DhL docked structure defined a unique binding site, in a similar position to the binding site of carozolol in the β-adrenergic receptor. The interactions are mainly of the hydrophobic type as the only observed hydrogen bond is with Asp 81 and a DhL carbonyl oxygen. The DhL triple ring was making hydrophobic and aromatic interactions within a 4-Å radius with Val 82, Cys 85, Thr 86, Leu 154, Tyr 160, Ile 161, Ser 164, Ser 168, Trp 208, Phe 212 and Phe 236 (Fig. 5). The results of quercetin′s docking showed that the lowest energy conformations were mainly grouped into a single cluster, at −8.14 binding energy, which defined a unique binding site. In this solution, the quercetin double ring is superposed to the DhL triple ring, while the single ring of quercetin is pointing towards the access channel (Fig. 6). The interactions of the quercetin double ring are also of the hydrophobic type, similar to the interactions held by the DhL triple ring. The quercetin lone ring only has a stacking interaction with the Phe 236 (3.6 Å) aromatic ring. Comparison of the energy of binding and inhibition constants of the docking runs can be seen in Table 1.

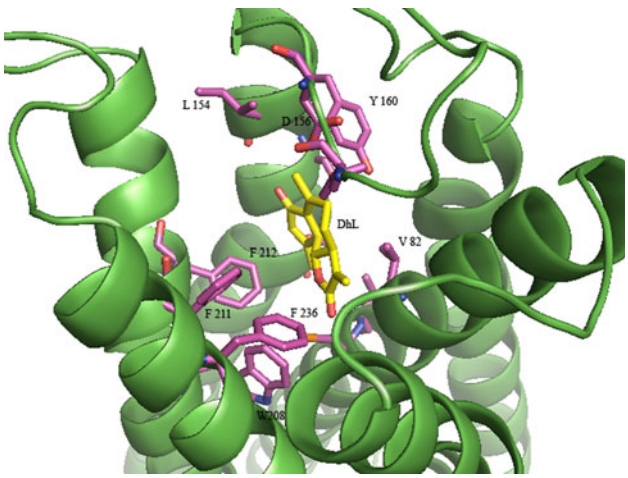

Fig. 5 Stick representation of DhL (*yellow*) bound in active site showing main interactions with receptor side chains (*magenta*). Amino acids are labeled using the *one letter* code

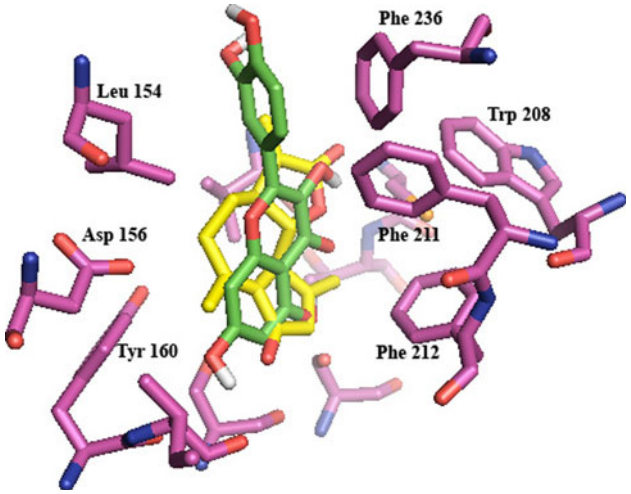

Fig. 6 Stick representation of DhL (*yellow*) and Quercetin (*green*) bound in the active site the α_2-adrenergic receptor, showing main interactions with the receptor side chains (*magenta*). Amino acids are labeled using the *three letter* code

Table 1 Comparison of the energy of binding and inhibition constants of the docking

Compound	Cluster	Binding energy	Inhibition constant
Dehydroleucodine	1_1(10)*	−7.38	3.87
Quercetin	1_1(9)*	−8.14	1.07
	2_1(1)*	−7.55	2.94

* Number of conformations in cluster

Discussion

Catecholamines inhibit propulsive contractions and reduce the tone of most intestinal smooth muscle. These effects appear to be mediated by both α- and β-adrenergic receptors. Activation of the β-receptor located on smooth muscle cells results in their relaxation. The α_2-adrenergic receptors mediate several physiological responses in the gastrointestinal tract, such as regulation of gastric and intestinal motility (Ruffolo et al. 1993) and secretions (DiJoseph et al. 1984). Several studies have shown that clonidine (α_2-receptor agonist) inhibits intestinal transit in rats (Ruwart et al. 1980) and mice (Jiang et al. 1988; Ramabadran et al. 1990), and has antidiarrheal effects in rats and mice (Lal et al. 1981; Spraggs and Bunce 1983; Doherty and Hancock 1983).

Our results demonstrated that DhL, in doses of 60 and 80 mg/kg, inhibits small intestinal motility in mice. These effects were not modified by prazosin, propranolol, atropine or naloxone. On the other hand the inhibitory effect of DhL on small intestinal motility was antagonized by phentolamine, an α_1, α_2-adrenergic receptor antagonis, and yohimbine, an α_2-adrenergic receptor antagonist. Consequently, these observations indicate a role for the α_2-adrenergic system in the action of DhL upon intestinal motility.

Natural products such as quercetin and some flavonoids significantly reduced intestinal transit in mice. Quercetin inhibits small intestinal motility in a dose-related manner (Meli et al. 1990). Yohimbine and phentolamine antagonized the effect of quercetin on intestinal transit, indicating a role for A2AR (Di Carlo et al. 1994).

The inhibition of the intestinal transit for DhL, which would indicate an interaction with the α_2-adrenergic receptor, was supported by the docking studies of DhL onto the homology model of the α_2-adrenergic receptor. Although homology models have shortcomings, usually generated in the available template, in our case, the target A2AR and the template, the human β_2-adrenergic receptor, belong to the same family of G protein coupled seven transmembrane receptors that binds similar sorts of substrates. AUTODOCK is the most used docking program with many successes in the field of drug design. In conclusion, the present results suggest that DhL produces an inhibitory effect upon intestinal motility, and its action may be mediated, at least in part, through the α_2-adrenergic receptor.

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