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In Silico Analysis of Sea Urchin Pigments as Potential Therapeutic Agents Against SARS-CoV-2: Main Protease (Mpro) as a Target.

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The SARS-CoV-2 outbreak has spread rapidly and globally generating a new coronavirus disease (COVID-19) since December 2019 that turned into a pandemic. Effective drugs are urgently needed and drug repurposing strategies offer a promising alternative to dramatically shorten the process of traditional de novo development. Based on their antiviral uses, the potential affinity of sea urchin pigments to bind main protease (Mpro) of SARS-CoV-2 was evaluated in silico. Docking analysis was used to test the potential of these sea urchin pigments as therapeutic and antiviral agents. All pigment compounds presented high molecular affinity to Mpro protein. However, the 1,4-naphtoquinones polihydroxilate (Spinochrome A and Echinochrome A) showed high affinity to bind around the Mpro´s pocket target by interfering with proper folding of the protein mainly through an H-bond with Glu166 residue. This interaction represents a potential blockage of this protease´s activity. All these results provide novel information regarding the uses of sea urchin pigments as antiviral drugs and suggest the need for further in vitro and in vivo analysis to expand all therapeutic uses against SARS-CoV-2.

In silico analysis of sea urchin pigments as potential therapeutic agents against SARS-CoV-2: Main protease (Mpro) as a target

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Abstract.

The SARS-CoV-2 outbreak has spread rapidly and globally generating a new coronavirus disease (COVID-19) since December 2019 that turned into a pandemic. Effective drugs are urgently needed and drug repurposing strategies offer a promising alternative to dramatically shorten the process of traditional *de novo* development. Based on their antiviral uses, the potential affinity of sea urchin pigments to bind main protease (Mpro) of SARS-COV-2 was evaluated *in silico*. Docking analysis was used to test the potential of these sea urchin pigments as therapeutic and antiviral agents. All pigment compounds presented high molecular affinity to Mpro protein. However, the 1,4-naphtoquinones polihydroxilate (Spinochrome A and Echinochrome A) showed high affinity to bind around the Mpro´s pocket target by interfering with proper folding of the protein mainly through an H-bond with Glu166 residue. This interaction represents a potential blockage of this protease´s activity. All these results provide novel information regarding the uses of sea urchin pigments as antiviral drugs and suggest the need for further *in vitro* and *in vivo* analysis to expand all therapeutic uses against SARS-CoV-2.

Keywords: 2019 pandemic; 1,4-naphtoquinones polihydroxilate; Spinochrome A; Echinochrome A; antiviral drug.

Introduction

The novel SARS-CoV-2 is an enveloped, positive-sense, single-stranded RNA virus belonging to the order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae* which has generated a new coronavirus disease (COVID-19) worldwide. Coronaviruses (CoVs) have the ability to infect multiple species with rapid change through recombination; this constitutes an ongoing threat to human health. Three betacoronaviruses have crossed the species barrier and produced deadly pneumonia in humans: severe acute respiratory syndrome coronavirus (SARS-CoV), Middle-East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2 causal agent of the COVID-19 pandemic. Coronaviruses code dozens of proteins, some of them involved in viral replication and entry into cells. As the most abundant protein in the betacoronaviruses virion structure, the main protease (Mpro, monomer between 25-30 kDa) is responsible for the structure and it is inserted into the envelope through three transmembrane domains. The amino constitutes a small ectodomain and can be modified by glycosylation which influences the tropism of the organs to be infected, such as the interferon-inducing capacity (IFN) of some coronaviruses. Therefore, the Mpro is considered the engine for the assembly of viral particles (Perrier et al., 2019), and it constitutes a suitable drug target since it is a key enzyme for coronavirus replication (Zhang et al., 2020a). The Mpro sequence is highly conserved within Coronaviruses. The viral replication can be inhibited in the active site in Mpro of SARS-CoV (Jin et al., 2020), which is located in the same position in SARS-CoV and SARS-CoV-2 between domain I (8-99 aa) and domain II (100-183 aa). Mpro presents flexibility in the binding site conformation constituting a good target for small drugs (Bzowka et al., 2020; Bzówka et al., 2020).

With the lack of available therapies and vaccines for COVID-19 treatment, scientists around the world have expanded and ramped up research on identifying promising inhibitors for preventive and supportive therapies. Drug repositioning is a recommended approach to face an unmet medical need for a new disease like COVID-19 (Pushpakom et al., 2018; Rosa and Santos, 2020). The molecular docking approach may predict the binding site on a complimentary basis in terms of the ligand and the target (Kitchen et al., 2004). Recent results of *in silico* docking analysis have indicated that certain positive bioactive compounds could be potent inhibitors of Mpro (Khaerunnisa et al., 2020; Pendyalaa and Patrasa, 2020).

Natural bioactive compounds are being extracted from a wide variety of sources, offering fewer side effects and accessible costs. Although research on marine natural products dates back more

than 50 years, only a few compounds have resulted in clinical trials and even fewer have been approved (Serive and Bach, 2018). A large number of pigments have photoprotection, anti-inflammatory and antioxidant effects, among other properties (Serive and Bach, 2018), leading to their use in cosmetics, functional food, nutraceutical and pharmaceutical products. Natural pigments incur no toxicity and that is why humans have been using them for clinical purposes. The relationship between food and health is known and well documented (Moniruddin, 2020; Syed, 2020).

Sea urchins are marine echinoderms that have been consumed by humans since ancient times (Rubilar and Crespi-Abril, 2017). In Asian culture, the sea urchin appears as far as long ago as in the "Materia Medica" of the Ming Dynasty author by Li Zhongli in 1647. In Chinese medicine, sea urchin roe is known for its benefits to the heart, bones, blood and also it counteracts impotence. Sea urchins can have different types of pigments such as carotenoids (astanxanthin, fucoxanthin and β-carotene) and 1,4-naphtoquinones polihydroxilate (commonly known as Spinochromes) (Cirino et al., 2017; Vasileva et al., 2017). Currently, there are now numerous studies that demonstrate the use of pigments to prevent cardiovascular and neurodegenerative diseases, as well as their antidiabetic, antiparasitic, anti-inflammatory, anti-obesity, anti-age-related macular degeneration, anticancer, and immunostimulatory effects (Serive and Bach, 2018). Nowadays the most common use of natural pigments is related to the nutraceutical and cosmeceutical industries. To reach the pharmaceutical market, bioactive pigments must satisfy pharmacokinetic descriptions, clinical studies, and regulation requirements.

In order to contribute to the identification of new drugs targeting the SARS-CoV-2 main protease and because sea urchin pigments are promising molecules, the aim of this *in silico* study was to evaluate the potential binding affinity of sea urchin pigments on Mpro through a docking analysis.

Methodology

Protein and ligands preparation

The receptor preparation was done according to Forli et al. (Forli et al., 2016) with modifications. The molecular 3D structure of SARS-CoV-2 Mpro protein co-crystalized with an inhibitor was obtained from Protein Data Base PDB (https://www.rcsb.org/): 6LU7,

105 resolution 2.16 Å (Kris-Etherton et al., 2002; Xu et al., 2020; Zhang et al., 2020b). Water and
106 ligand molecules were removed from the file and the software AutoDockTools (ADT version
107 1.5.7) was used for receptor and ligands preparation. Polar hydrogens were added and the partial
108 Kollman charges were assigned to the proteins.

109 The co-crystallized N3 ligand was extracted from the PDB structure and polar hydrogens and
110 Gasteiger charges were added through the ADT software. The prepared structure was saved in
111 .pdbqt format.

112 The SMILE of sea urchin pigments Spinochomre A, Echinochrome A, β-carotene, astaxanthin
113 and fucoxanthin and drugs Carmofur, Cinanserin, Disulfiram, Ebselen, PX-12, Shikonin,
114 TDZD-8 and Tideglusib were downloaded from Chemical Entities of Biological Interest
115 (ChEBI) and PubChem (https://pubchem.ncbi.nlm.nih.gov/), transformed to PDB and polar
116 hydrogens and Gasteiger charges were added and saved in .pdbqt format by ADT software. In
117 the case of Ebselen the selenium atom was changed for a sulfur because otherwise it was not
118 possible to perform the molecular docking analysis.

119

120 *Molecular docking*

121 The docking simulations were performed using AutoDock vina 1.1.2 (Trott and Olson, 2010).
122 The center of the search space for Mpro dockings (-9.732, 11.403, 68.483) have been
123 determined on the basis of the co-crystallized bound N3 ligand, and its size has been set to
124 20x20x20 Å to cover the active site of the protease. The exhaustiveness has been set to 24 in
125 all docking analyses while the remaining AutoDock Vina parameters have been kept at default
126 values. The results of the docking experiments have been ranked according to their Vina score
127 and docking poses were visually inspected with UCSF Chimera software (Pettersen et al.,
128 2004).

129 In the case of Echinochrome A, the top ranked candidates were selected for further analysis of
130 protein-ligand interactions. Hydrogen bonds (H-bonds) were detected with UCSF Chimera
131 relax H-bonds constraints (0.5 Å and 25°). All direct interactions were also identified as clashes
132 and contact. Note that clashes are unfavorable interactions where atoms are too close together,
133 with contacts denoting all kinds of direct interactions (polar and nonpolar, favorable and
134 unfavorable), including clashes.

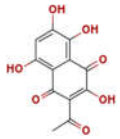
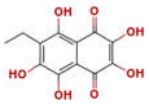
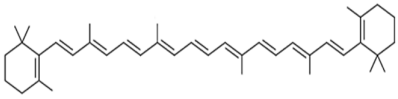
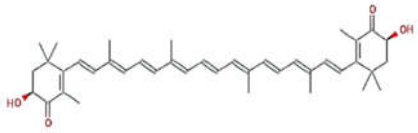
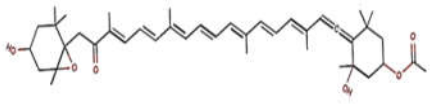
135

As a validation protocol for Mpro analysis, the co-crystallized N3 peptide was removed and redocked with the substrate-binding site of SARS-CoV-2 Mpro (6LU7) by using the same docking parameters. The generated re-docked pose was quite similar to the co-crystalized conformation (RMSD 5.496 Å).

Results and Discussion

This study was focused on the potential of sea urchin pigments as antiviral drugs by inhibiting Mpro activity since they are small molecules (Table 1). These pigments share the common property of being antioxidant molecules. Carotenoids have different common biological functions due to their chemical structure. They are characterized by the covalent chemical bonding of polyene units, constituting a skeleton of at least 40 carbon atoms with conjugated double bonds which provide an extensive cloud of pi electrons that interact with free radicals conferring their antioxidant capacity (Galasso et al., 2017; Young and Lowe, 2018). This family of pigments includes carotenes and xanthophylls. Carotenes, such as β-carotene, have only carbon and hydrogen atoms and are therefore hydrophobic while xanthophylls, such as astaxanthin and fucoxanthin, have oxygen in their terminal rings, making them somewhat more polar than carotenes and enhancing their antioxidant properties (Galasso et al., 2017). The 1,4-naphtoquinones polyhydroxylated, such as Spinochrome A (SpinA) and Echinochrome A (EchA) have a chemical structure that include several hydroxylated groups which are appropriate for free-radical scavenging, diminishing ROS and preventing redox imbalance (Jeong et al., 2014). The position of OH groups and number in the quinoid fragment may be important since the OH groups in polyhydroxylated 1,4-naphthoquinones in the R1, R2, and R5 positions play key roles in both iron-ion complexing and free radical scavenging (Lebedev et al., 2008).

166 Table 1. Potentially bioactive pigments in sea urchins.

N°	Compound Name	IUPAC Compound Name	2D Lineal Structure	Molecular Weight (g mol^{-1})
1	**Spinochrome A**	1,4-Naphthalenedione, 2-acetyl-3,5,6,8-tetrahydroxy 6-acetyl-4,5,7,8-tetrahydroxynaphthalene-1,2-dione 2-Acetyl-3,6-dihydroxynaphthazarin Spinochrome A *1. o (Yabuzaki.J 2015)*	 *(2) o (©ChemExper Inc)*	264.19
2	**Echinochrome A**	6-ethyl-2,3,5,7,8-pentahydroxy-1,4-Naphthoquinone, 6-ethyl-2,3,5,7,8-pentahydroxy-1,4-Naphthalenedione, 6-Ethyl-2,3,7-trihydroxynaphthazarin *(4) o (©ChemExper Inc)*	 *(3) o (PubChem®)*	266.22
3	**β-Carothene**	1,3,3-Trimethyl-2-[(1E,3E,5E,7E,9E,11E,13E,15E,17E)-3,7,12,16-tetramethyl-18-(2,6,6-trimethylcyclohexen-1-yl)octadeca-1,3,5,7,9,11,13,15,17-nonaenyl]cyclohexene [5] o S. Kim et al., 2016	 (6) ohttps://hmdb.ca	536.87
4	**Astaxanthine**	(6S)-6-hydroxy-3-[(1E,3E,5E,7E,9E,11E,13E,15E,17E)-18-[(4S)-4-hydroxy-2,6,6-trimethyl-3-oxocyclohexen-1-yl]-3,7,12,16-tetramethyloctadeca-1,3,5,7,9,11,13,15,17-nonaenyl]-2,4,4-trimethylcyclohex-2-en-1-one (7) o (S. Kim et al., 2016)	 (7) o *(PubChem®)*	596.80
5	**Fucoxanthin**	[(1S,3R)-3-hydroxy-4-[(3E,5E,7E,9E,11E,13E,15E)-18-[(1S,4S,6R)-4-hydroxy-2,2,6-trimethyl-7-oxabicyclo[4.1.0]heptan-1-yl]-3,7,12,16-tetramethyl-17-oxooctadeca-1,3,5,7,9,11,13,15-octaenylidene]-3,5,5-trimethylcyclohexyl] acetate *(9) o (PubChem®)*	 *(10) o (PubChem®)*	658.90

The ability of these molecules to interact with SARS-CoV-2 main protease (Mpro) was analyzed. Recently, Jin et al (Jin et al., 2020) identified new drugs that are able to inhibit this enzyme and hence they were included for comparison. The binding energies obtained from molecular docking analysis of Mpro with sea urchin pigments and known drugs are shown in Figure 1. All pigments presented low binding energy which indicates a higher affinity for the viral protein than the rest of the tested compounds (Vina docking scores, from -7.5 to -6.0 Kcal.mol^{-1}) with the exception of Astaxanthin (Vina docking score -3.0 Kcal.mol^{-1}). Only Tideglusib showed a higher binding energy than the other tested pigments (Vina docking scores, -7.9 Kcal.mol^{-1}). However, Tideglusib is a drug used in Alzheimer's disease and its ingestion can cause mild-moderate adverse reactions, as transient increases in serum creatine kinase, ALT—or gGT—diarrhea, nausea, cough, fatigue, and headache (Del Ser et al., 2013).

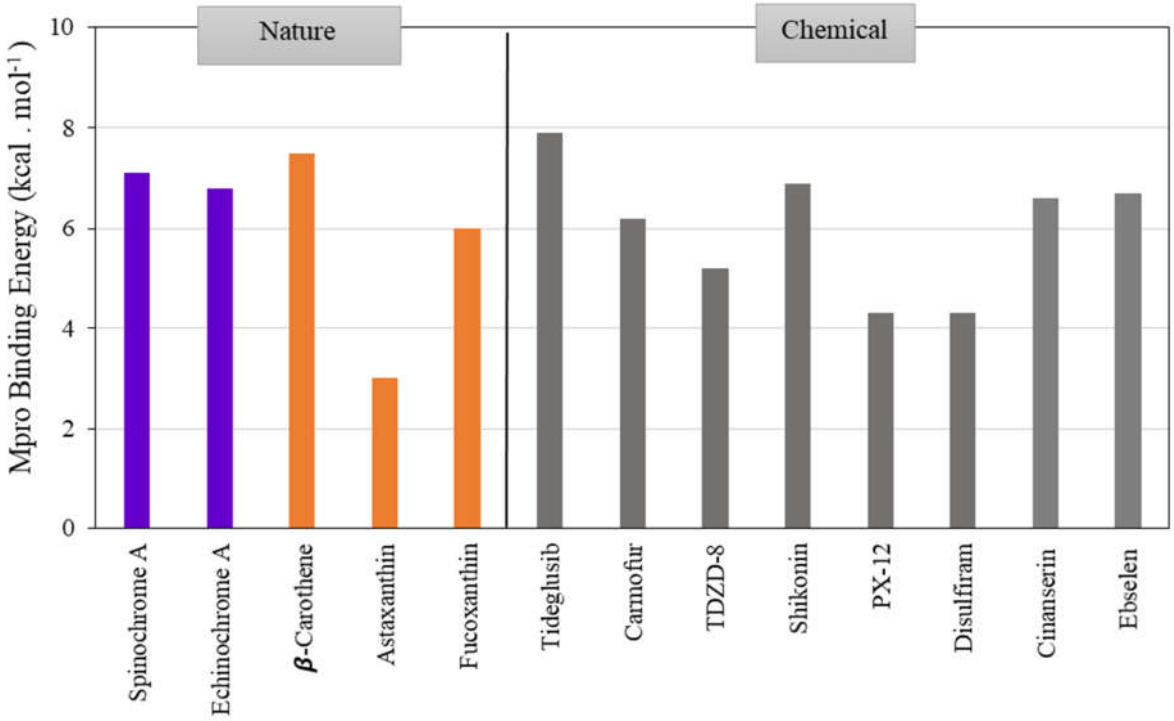


Figure 1. Mpro Binding energies of sea urchin pigments (1,4-naphtoquinones polyhydroxylated in purple; carotenoids in orange) and chemical compounds (in grey) and tested drugs.

Because of Mpro binding site flexibility (Bzowka et al., 2020; Bzówka et al., 2020) and considering that SpinA and EchA are small molecules with high affinity for this protein, the molecular interaction in the binding site was further evaluated. Both spinochromes have high affinity for the viral proteins (Vina docking scores, SpinA: -7.1 Kcal.mol^{-1}, EchA: -6.8 Kcal.mol^{-1}), which suggests they are potential antiviral drugs. The best docking pose of SpinA involved seven H-bonds with amino acids S144, C145, H163, E166 and L141, no clashes and 42 Van der Waals (VdW) contacts with G143, S144, L141, E166, M49, H163, C145, M165, Q189 and H41 (Figure 2). The best docking pose of EchA involved four H-bonds with amino acids S144 and E166, 51 VdW contacts with S144, C145, M165, Q189, E166, L141, M49, G143, H163, F40, H41, and N142 and no clashes (Figure 3). Mpro presents a homodimer with the pocket of the substrate-binding site formed by the interaction of Glu166 from one monomer with Ser1 from the other through an H-bond (Jin et al., 2020; Zhang et al., 2020b). Since EchA is a highly polar molecule, due to the presence of numerous hydroxyl groups, the bond between the Glu166 and EchA may interfere with the dimer interface. This is extremely important because the Mpro dimerization is essential for catalysis (Cheng et al., 2010), and if EchA is able to interfere in this matter it may inactivate Mpro.

Spinochromes have been found to have cardioprotective activity against the cytotoxicity of doxorubic (Yoon et al., 2019) and antiallergic effects (Pozharitskaya et al., 2013). However, there is no pharmaceutical products based on SpinA available in the market yet. On the other hand, Echinochrome A (EchA), has satisfied all pharmaceutical requirements and products made from this pigment, have been approved. EchA is the active compound of Histochrome™ and Gistochrome™, two Russian preparations for cardiopathies and glaucoma diseases. There is a large amount of literature regarding EchA uses (Jeong et al., 2014; Lebed'ko et al., 2015; Oh et al., 2019; Vasileva et al., 2017). The pharmacological activity observed in patients with various health issues, together with the identified low toxicity profiles, strongly support the potential and therapeutic benefits of these natural pigments for the treatment of various human diseases, particularly inflammation, cardioprotection and diabetes (Shikov et al., 2018).

Moreover, in animal models EchA has shown a wide range of biological properties, expanding possible therapeutic applications. For example, treatment with EchA in a neonatal murine model was able to prevent pulmonary fibrosis by reducing bleomycin-induced oxidative stress (Lebed'ko et al., 2015). In another study, mice with inflammatory bowel disease that were treated with EchA showed a reduced mortality and modulated the immune response, reducing inflammation and allowing tissue repair (Oh et al., 2019). In regard to COVID-19, the most

important application is the in vitro antiviral evidences of EchA against certain types of human viruses, such as tick-borne encephalitis virus (TBEV) and herpes simplex virus type 1 (HSV-1) (Fedoreyev et al., 2018). In these studies, infected cells were treated with EchA in combination with the antioxidants ascorbic acid and α-tocopherol in a ratio of 5:5:1, proving to be a mixture with powerful antiviral effects (Fedoreyev et al., 2018). The combination of EchA and the other antioxidants was able to neutralize virus infection, probably preventing the adsorption of the virus to the host cell receptors or causing damage in the viral capsid protein as it has been reported in other cases (Astani and Schnitzler, 2014; Fedoreyev et al., 2018; Garrett et al., 2012; Torky and Hossain, 2017). The antiviral activity of EchA with ascorbic acid and α-tocopherol has been hypothesized also to be a result of the interference with the redox imbalance normally caused by these viruses, resulting in no cytotoxicity (Di Sotto et al., 2018). EchA has the ability to improve oxygen supply to peripheral tissues and due to its antioxidant power it protects the mitochondria, improving the rate of oxygen consumption, the production of ATP and the regulation of the transcription of some genes (Vasileva et al., 2017). As a consequence, EchA acts directly, either alone or in combination, on virus particles by inactivating them, and also acts indirectly improving antioxidant defense mechanisms of the host cell.

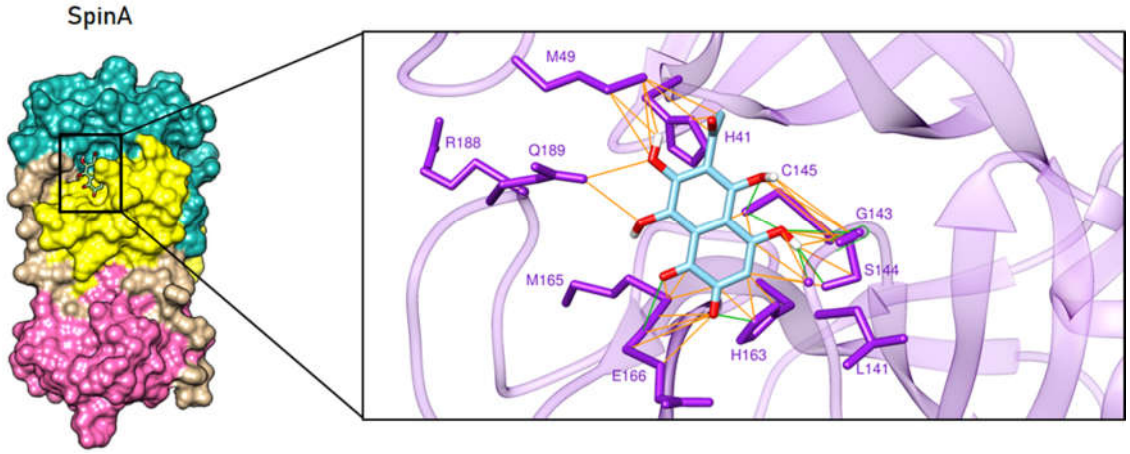


Figure 2. One protomer of SARS-CoV-2 Mpro interaction with SpinA presents the best docking pose. On the left, SpinA is shown binded with the Mpro binding pocket. Mpro domains are shown in different colours, Domain I (residues 8-101) in aquamarine, Domain II (residues 102-184) in yellow and Domain III (residues 201-303) in pink. On the left, there is a zoomed view of the substrate-binding pocket with the best docking pose of SpinA. The key residues forming the binding pocket are shown in sticks and labeled.

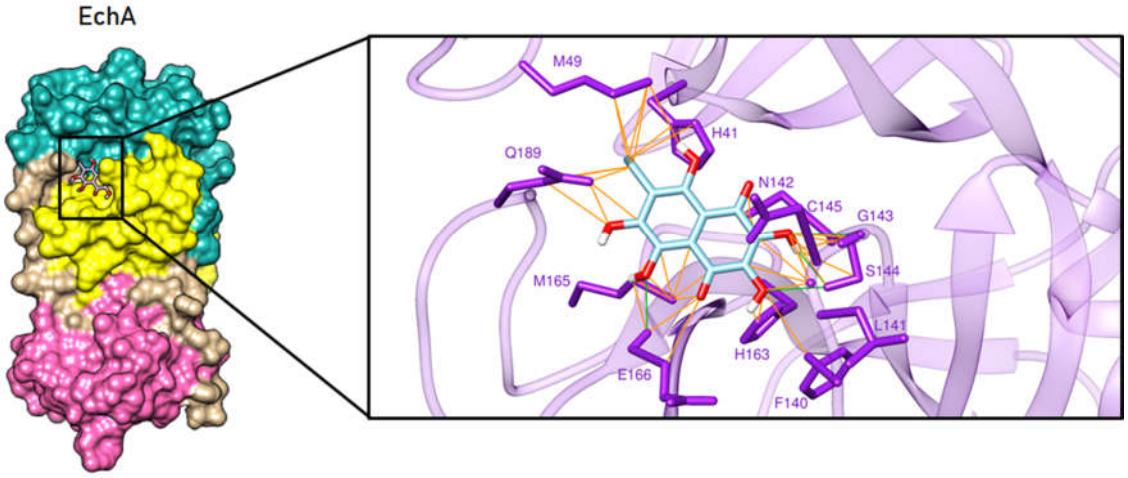


Figure 3. One protomer of SARS-CoV-2 Mpro interaction with EchA best docking pose. On the left EchA is shown binded with the Mpro binding pocket. Mpro domains are shown in different colours, Domain I (residues 8-101) in aquamarine, Domain II (residues 102-184) in yellow and Domain III (residues 201-303) in pink. On the left, there is a zoomed view of the substrate-binding pocket with the best docking pose of EchA. The key residues forming the binding pocket are shown in sticks and labeled.

EchA is already used as a drug therapy in humans and it also showed a good binding affinity to Mpro. The three-dimensional structures of Mpro of SARS-CoV and SARS-CoV-2 are quite similar. Both enzymes have a half-site activity and they only differ in a few amino acids and SARS-CoV-2 Mpro enzyme has a slightly higher catalytic activity (Jin et al., 2020; Zhang et al., 2020b). Moreover, substrate-binding pockets and substrate specificity from different CoV Mpro enzymes are conserved and suggest that targeting this site may lead to broad-spectrum inhibitors (Hegyi and Ziebuhr, 2002; Jin et al., 2020). Therefore, *in vitro* testing of Mpro of SARS-CoV and SARS-CoV-2 by EchA is necessary to confirm the inhibitory capacity.

Conclusion

In this *in silico* study, we used molecular docking to evaluate the potential interaction of natural sea urchin pigments with the Mpro protein of SARS-CoV-2. All pigment compounds presented a high molecular affinity to Mpro protein. However, EchA, a sea urchin pigment belonging to the family 1,4-naphtoquinones polihydroxilate, interacted with very high affinity to Mpro binding site. This may be related to its small size and the H-bond interactions between OH groups of EchA and Glu166 probably blocking the activity of Mpro. EchA is a natural marine pigment found in the test and spines of most sea urchin species in low concentrations. However,

it is highly concentrated in the eggs of the sea urchin *Arbacia dufresnii* in aquaculture systems and hence it is available from a natural marine source. All these results provide novel information regarding sea urchin pigments as antiviral drugs against SARS-CoV-2, suggesting the need for further analysis to expand therapeutic uses.

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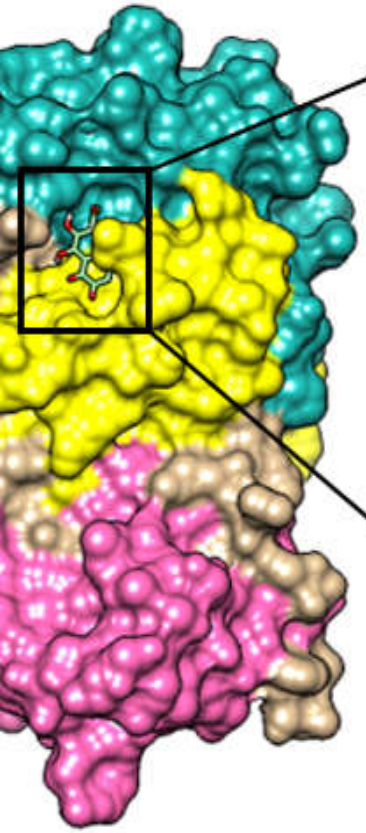

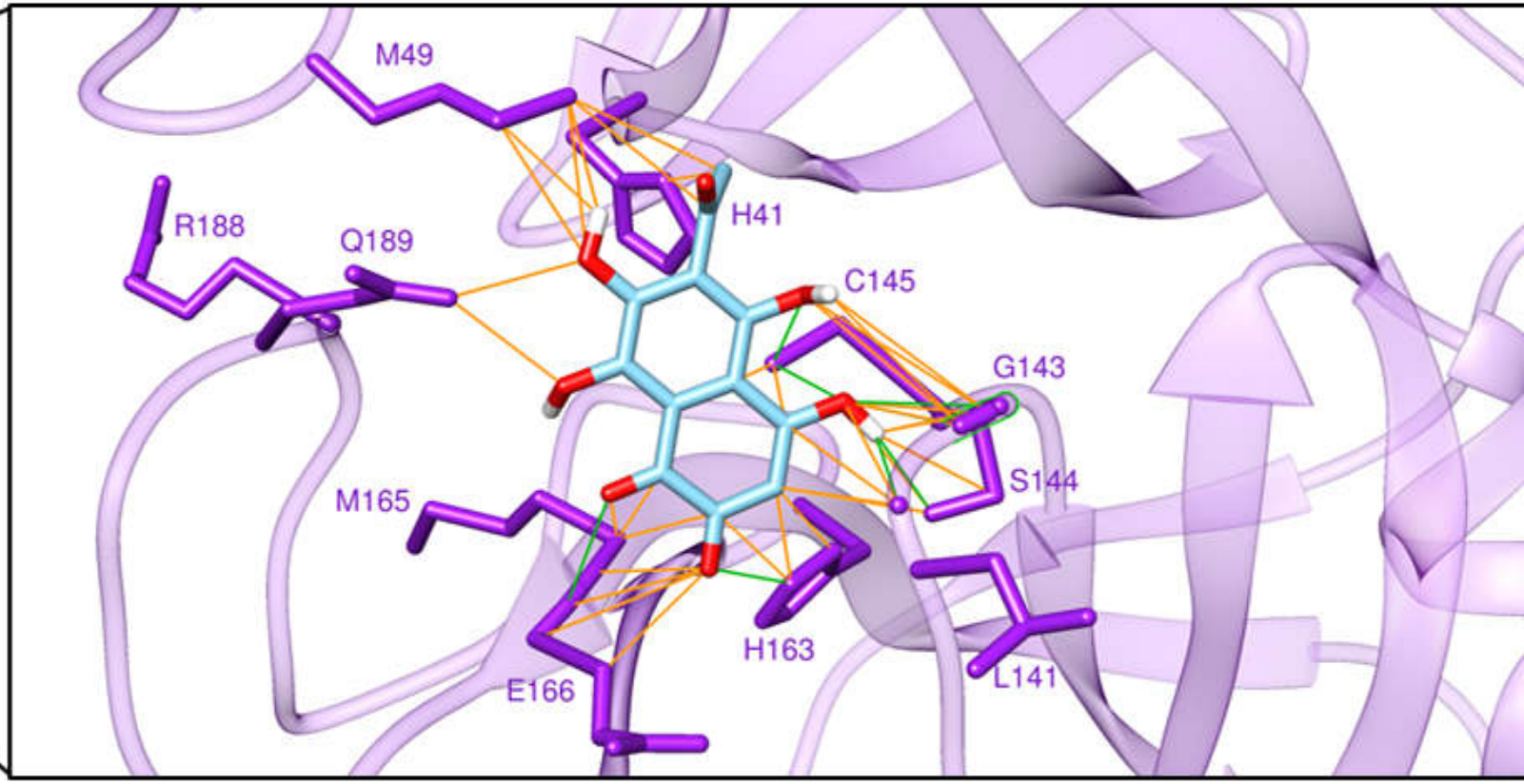

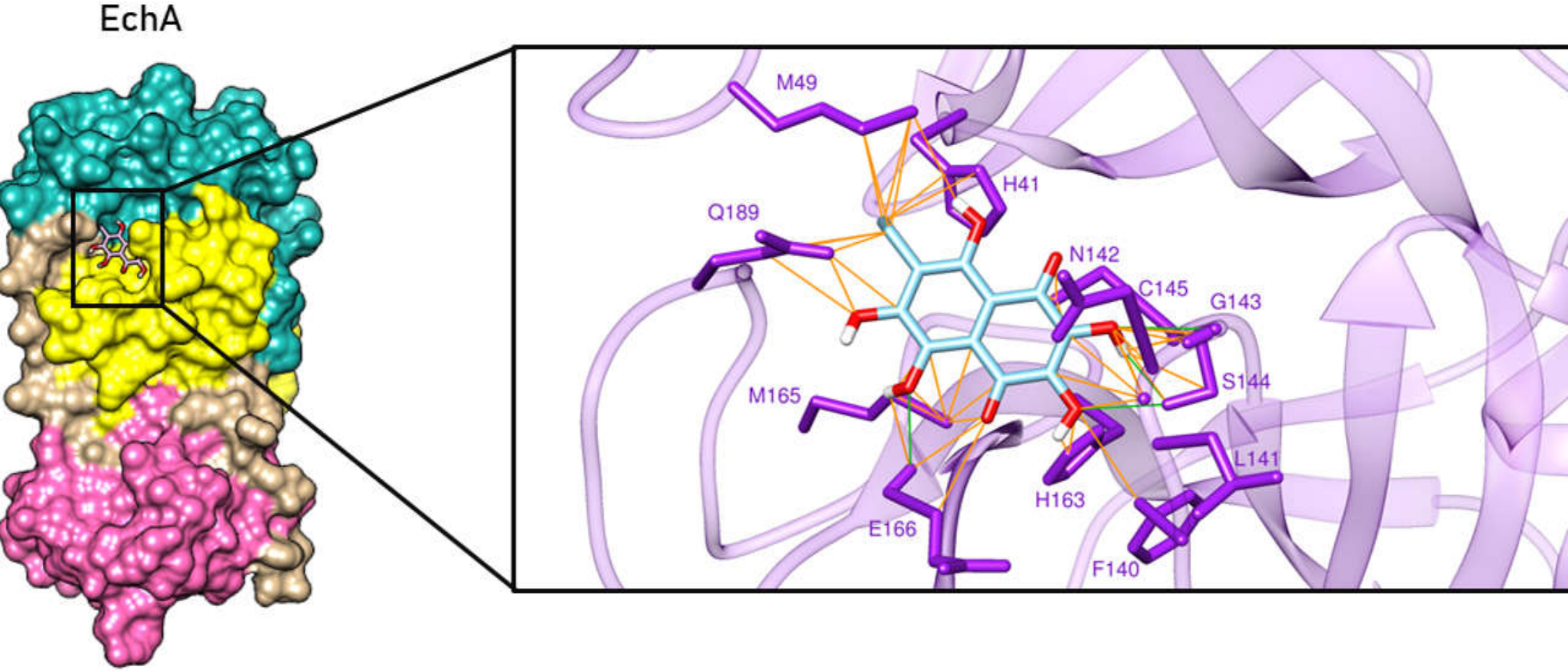
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