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Short communication

Carlina oxide inhibits the interaction of SARS-CoV-2 S glycoprotein with angiotensin-converting enzyme 2

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ABSTRACT
Carlina acaulis plant is a potential target for the industrial production of phytochemicals that display applicability in pharmacy and medicine. The dry roots of C. acaulis contain up to 2 % of essential oil, the main component (up to 99 %) of which is carlina oxide [2-(3-phenylprop-1-ynyl)furan]. This compound shows multidirectional biological activity, including antibacterial and antifungal properties. Here, we evaluated the capacity of carlina oxide to inhibit the interaction between SARS-CoV-2 and its human receptor in vitro and in silico. A bioluminescent immunoassay was used to study the interaction between the receptor binding domain (RBD) of viral spike protein and the human angiotensin-converting enzyme 2 (ACE2), which serves as a receptor for viral entry. A dose-effect relationship was demonstrated, and a concentration of carlina oxide causing half-maximal inhibition (IC50) of the RBD:ACE2 interaction was determined to be equal to 234.2 µg/mL. Molecular docking suggested the presence of carlina oxide binding sites within the RBD and at the interface between RBD and ACE2. Finally, this study expands the list of potential applications of C. acaulis as a crop species.

1. Introduction

Carlina acaulis L. constitutes a valuable source of bioactive compounds, with leaves of the plant being particularly rich in polyphenols and triterpenes (Strzemski et al., 2017b) and roots containing significant amounts of a polyacetylene species known as carlina oxide (Chalchat et al., 1996; Strzemski et al., 2020, 2016). Carlina oxide has been shown to suppress growth of cancer cells in culture (Wnorowski et al., 2020). Its application as a biopesticide has also intensively investigated (Benelli et al., 2022, 2020; Rizzo et al., 2021). Recent agricultural experiments revealed that up to 10 kg of carlina oxide can be harvested from 10,000 m² (one hectare) of cultivation (Strzemski et al., 2021), emphasizing the feasibility of large-scale production. This is of particular interest, as novel applications of carlina oxide emerge. Here, we hypothesized that carlina oxide may inhibit the interaction between spike protein of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and its human receptor – angiotensin-converting enzyme 2 (ACE2).

SARS-CoV-2 first emerged in Wuhan, China, in mid-December 2019, and from a local epidemic it spread all over the world, causing a COVID-19 pandemic. The disease claimed more than 6 million lives worldwide (Johns Hopkins Coronavirus Resource Center, 2022). For this reason, intensive work is underway to develop vaccines against SARS-CoV-2 (Thanh Le et al., 2020), with several of them already in widespread use (Baden et al., 2020; Polack et al., 2020). In parallel, multiple drug discovery and repurposing programs were lunched to identify inhibitors of SARS-CoV-2 infection (Dittmar et al., 2021; Owen et al., 2021), with several potential hits among herbal medicines and plant-based products (Jan et al., 2021).

The assessment of the interaction of potential drugs with the surface S glycoprotein of the SARS-CoV-2 virus constitutes a promising

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approach for identification of new inhibitors of the viral infection. The receptor-binding domain (RBD) of S glycoprotein is responsible for the interaction with ACE2 receptors on the surface of host cells and, consequently, for the viral entry (Ou et al., 2020; Walls et al., 2020). Here, we assessed the capacity of carlina oxide to inhibit the RBD:ACE2 interaction.

2. Materials and methods

2.1. Reference standards and chemicals

The carlina oxide of 96.2 % purity was obtained by distillation of C. acaulis roots in the Deryng apparatus. The identity and purity of the compound were confirmed in accordance with previously established methodology (Strzemski et al., 2017a), and the results of the analyses were reported earlier (Wnorowski et al., 2020). Because the carlina oxide content in the distilled oil was above 95 % (purity level common for many chromatographic standards), the natural product was simply referred to as “carlina oxide” later in the manuscript.

2.2. Biological material

Blood was collected from volunteers in anticoagulant-free tubes for serum preparation. The total number of four vaccinated individuals volunteered for the study. Also, pre-Covid-19 samples from randomly selected six patients were analyzed. There were only two groups in the analysis (vaccinated and pre-Covid-19). There was no mixing of material from multiple individuals. The research was conducted with the consent of the Bioethics Committee of the Medical University of Lublin no. KE0254/244/2020.

2.3. Protein-protein interaction assay

Table 1

| Binding site                  | Residues involved in binding | Calculated binding energy [kcal/mol] |
|------------------------------|------------------------------|--------------------------------------|
| RBD/ACE2 interface           | ACE2: Asp30, His34, Glu37, Asp38, | -5.1                                 |
| (yellow pose at Fig. 4A)    | Lys353 RDB: Arg303, Glu406,  |
|                             | Gln409, Lys417, Ile418, Tyr453, |                                     |
|                             | Leu653, Gln493, Ser494, Tyr495, |                                     |
|                             | Gly496                          |                                     |
| Site 1 within RBD            | RBD: Phe338, Val341, Phe342,   | -7.6                                 |
| (blue pose at Fig. 4A)      | Lys356, Ile358, Ala363, Tyr365, |                                     |
|                             | Leu368, Tyr369, Phe377, Cys379, |                                     |
|                             | Cys432, Leu387, Val395, Ala397, |                                     |
|                             | Ile434, Leu513, Phe515          |                                     |
| Site 2 within RBD            | RBD: Phe338, Gly339, Phe342,   | -5.7                                 |
| (light blue pose at Fig. 4A) | Asn343, Val367, Leu368, Ser371, |                                     |
| Site 3 within RBD            | RDB: Leu368, Tyr369, Ala372,   | -5.6                                 |
| (green pose at Fig. 4A)     | Ser373, Phe374, Trp436          |                                     |
| Site 4 within RBD            | RDB: Pro426, Asp428, Phe429,   | -5.1                                 |
| (cyan pose at Fig. 4A)      | Thr430, Phe464, Asn394, Tyr396, |                                     |
|                             | Ser514, Glu516                  |                                     |

Fig. 1. Serum samples from vaccinated individuals suppress RBD:ACE2 interaction. The mean difference in RBD:hACE protein-protein interaction (%) between pre-Covid19 serum samples and serum samples from vaccinated individuals (three doses of mRNA vaccine) is shown in the above Gardner-Altman estimation chart. Values for ‘Control’ and ‘Carlina Oxide’ groups were plotted on the left vertical axis; the mean difference was plotted on a right floating axis as a bootstrap sampling distribution. The mean difference was illustrated as a black dot, whereas the 95 % confidence interval was indicated by the ends of the vertical error bar. Unpaired mean difference between groups = -95.7 [95.0 % CI - 1.03e+02, -89.0]. P-value < 0.0001.

Fig. 2. Carlina oxide inhibits RBD:hACE2 interaction. The mean difference in RBD:hACE protein-protein interaction (%) between vehicle control (DMSO) and carlina oxide (300 µg/mL) is shown in the above Gardner-Altman estimation chart. Values for ‘Control’ and ‘Carlina Oxide’ groups were plotted on the left vertical axis; the mean difference was plotted on a right floating axis as a bootstrap sampling distribution. The mean difference was illustrated as a black dot, whereas the 95 % confidence interval was indicated by the ends of the vertical error bar. Unpaired mean difference between groups = -67.7 [95.0 % CI - 72.6, - 62.0]. P-value = 0.0008.

Fig. 3. Carlina oxide inhibits RBD:hACE2 protein-protein interaction in a dose-dependent fashion. The four-parameters sigmoidal curve was fitted to control-normalized RBD:hACE2 interaction values. The calculated IC50 was equal to 234.2 µg/mL.

oxide content in the distilled oil was above 95 % (purity level common for many chromatographic standards), the natural product was simply referred to as “carlina oxide” later in the manuscript.

2.2. Biological material

Blood was collected from volunteers in anticoagulant-free tubes for serum preparation. The total number of four vaccinated individuals volunteered for the study. Also, pre-Covid-19 samples from randomly selected six patients were analyzed. There were only two groups in the analysis (vaccinated and pre-Covid-19). There was no mixing of material from multiple individuals. The research was conducted with the consent of the Bioethics Committee of the Medical University of Lublin no. KE0254/244/2020.

2.3. Protein-protein interaction assay

Strength of the interaction between the viral RBD with human ACE2...
Fig. 4. Interactions of carlina oxide with human RBD of SARS-CoV-2 trimer spike. (A) Carlina oxide binding sites (green, light blue, blue, cyan - rendered in ball mode) are located within the RBD of SARS-CoV-2 trimer spike and at the interface between RBD and ACE2 (yellow; rendered in ball mode). The energetically lowest orientation within the RBD (blue, rendered in stick mode) and energetically higher orientation at the interface between RBD and ACE2 (yellow, rendered in stick mode) were selected and shown with residues involved in binding (rendered in stick mode, element color code). The residues important for interaction with other possible poses of carlina oxide (green, light blue, and cyan) are listed in Table 1. All non-polar hydrogen atoms are hidden. (B-C) 2D views depicting interactions of carlina oxide within RBD of SARS-CoV-2 spike (panel B) and at the interface between RBD and human ACE2 (panel C).
was assessed using Lumit SARS-CoV-2 spike RBD:hACE2 immunoassay (Promega) according to the manufacturer’s protocol (Alves et al., 2021). The assay is based on the immunodetection and luciferase complementation. In brief, carlina oxide, serum samples or appropriate vehicle were incubated with RBD tagged with Fc-domain from rabbit. Next, ACE2 conjugated with murine Fc-domain was added. Subsequently, secondary antibodies against rabbit and mouse were added. The secondary antibodies were bearing splittable luciferase enzyme that would complement when in proximity. The mix was incubated for 60 min at room temperature. Finally, luciferase substrate was provided for 30 min and the generated luminescence was recorded using Synergy H1 plate reader (BioTek).

2.4. Molecular docking

The human ACE2-bound SARS-CoV-2 spike at 3.64 Å atomic resolution (PDB ID:7KMS) was used for molecular docking of carlina oxide (Zhou et al., 2020). Carlina oxide structure was downloaded from PubChem database (PubChem CID 164634) and geometrically optimized using the semi-empirical AM1 method found in Spartan 10 V.1.1.0 (Wavefunction, Inc., Irvine, CA, USA), as previously indicated (Targowska-Duda et al., 2019). AutoDock Vina (Trott and Olson, 2010) was used for docking simulations of flexible ligand into the RBD of SARS-CoV-2 spike protein. The grid box was generated using MGL-AutoDockTools 1.5.6. The selected dimension for the grid maps was 40 Å × 40 Å × 40 Å, with a grid-point spacing of 1 Å, to cover the RBD with the interface between RBD and ACE2. The AutoDock Vina parameters were exhaustiveness (570) and number of modes (20) as previously reported (Targowska-Duda et al., 2019). The energetically lower orientations were exported from each cluster of superposed poses for carlina oxide at each binding site and included in the Table 1.

2.5. Statistical analysis

Differences between two groups of data were statistically assessed using estimation statistics outline (Ho et al., 2019). Data are presented on Gardner-Altman estimation plots. The effect sizes and CIs were reported as: effect size [95.0% CI upper bound; upper bound]. Exactly 5 × 10^3 bootstrap samples were taken. The confidence interval was bias-corrected and accelerated. The P-values reported are the likelihoods of observing the effect sizes, if the null hypothesis of zero difference is true. Precisely 5 × 10^3 reshuffles of the control and test labels were performed for each permutation P-value.

Dose-response curve was generated using Prism v8.4.3 (GraphPad Software) by fitting sigmoidal curve equation to obtained datapoints. The concentration of carlina oxide causing 50% inhibition (IC50) of the protein-protein interaction was calculated.

3. Results and discussion

A bioluminescent immunoassay was used to study the interaction between SARS-CoV-2 spike RBD and human ACE2. To validate the assay, RBD was pre-incubated with either pre-pandemic serum samples (n = 6) or with serum samples obtained from patients subjected to three doses of anti-Covid mRNA vaccine (n = 4) (Fig. 1). Although a limited number of samples was used in the assay, a significant decrease (P-value < 0.0001) in the relative RBD:hACE2 protein-protein interaction in samples from vaccinated individuals compared to naïve patients was observed, presumably to the presence of neutralizing antibodies. The unpaired mean difference between pre-Covid19 and vaccine samples was −95.7 [95.0% CI −1.03e−02, −89.0].

Surprisingly, a suppression of RBD:hACE2 protein-protein interaction was observed in the presence of carlina oxide (Fig. 2). The difference in the percentage of luminescent signal between the samples pre-incubated with vehicle (DMSO) control and carlina oxide was −67.7 [95.0% CI −72.6, −62.0]. The P-value of the two-sided permutation t-test was 0.0008.

In order to confirm that the inhibition of RBD:hACE2 interaction elicited by carlina oxide occurs in a dose-response fashion, RBD was pre-incubated with increasing doses of carlina oxide (Fig. 3). The concentration causing half-maximal inhibition (IC50) was determined to be equal to 234.2 µg/mL.

To get more insight into the molecular mechanism of the identified inhibitory action of carlina oxide, a molecular docking was conducted. Molecular docking results indicated the locations of possible binding sites and structural components for carlina oxide at RBD of SARS-CoV-2 spike model. Carlina oxide interacted on several binding sites within the RBD and RBD/ACE2 interface. In particular, the energetically lowest site was located within the RBD where the ligand interacted mostly by hydrophobic interactions (Fig. 4A and B). Carlina oxide may also bind at the interface between RBD and ACE2 as presented in Table 1 and Fig. 4A and C.

4. Conclusions

C. acaulis plants are potential target for the industrial production of phytochemicals that display applicability in pharmacy and medicine. Here, we demonstrated for the first time that carlina oxide, one of the biologically active constituents of the C. acaulis, displays in vitro inhibitory activity towards RBD:ACE2 interaction. This study extends the list of potential applications of C. acaulis as a crop species. However, further studies are required to confirm that carlina oxide blocks viral entry and limits SARS-CoV-2 infectivity in vivo.

CRediT authorship contribution statement

Maciej Strzemski, Artur Wnorowski: Conceptualization, Artur Wnorowski, Katarzyna Targowska-Duda, Maciej Strzemski: Methodology. Artur Wnorowski, Katarzyna Targowska-Duda: Software, Artur Wnorowski, Katarzyna Targowska-Duda: Validation, Artur Wnorowski, Katarzyna Targowska-Duda, Sylwia Wnorowska, Jacek Kurzepa, Maciej Strzemski: Formal analysis. Sylwia Wnorowska, Katarzyna Targowska-Duda, Maciej Strzemski: Investigation. Artur Wnorowski, Katarzyna Targowska-Duda, Sylwia Wnorowska: Data curation, Artur Wnorowski, Katarzyna Targowska-Duda, Sylwia Wnorowska: Writing – original draft. Artur Wnorowski, Katarzyna Targowska-Duda, Maciej Strzemski: Writing – review & editing. Artur Wnorowski, Katarzyna Targowska-Duda: Visualization. Jacek Kurzepa: Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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