Active Components of Essential Oils as Anti-Obesity Potential Drugs Investigated by in Silico Techniques

**ABSTRACT:** In this study, for the first time, we have considered essential oils (EOs) as possible resources of carbonic anhydrase inhibitors (CAIs), in particular against the mitochondrial isoform VA that, actually, represents an innovative target for the obesity treatment. In silico structure-based virtual screening was performed in order to speed up the identification of promising antiobesity agents. The potential hit compounds were submitted to in vitro assays and experimental results, corroborated by molecular modeling studies, showed EOs components as a new class of CAIs with a competitive mechanism of action due to the zinc ion coordination within the active sites of these metallo-enzymes.

**KEYWORDS:** essential oils, antiobesity, human carbonic anhydrase, inhibition, virtual screening

**INTRODUCTION**

Obesity is ranked by the World Health Organization (WHO) as one of the top 10 global health problems, usually correlated with other pathologies, such as cardiovascular and musculoskeletal disorders, type 2 diabetes, depression, and several cancers including colon, breast, and endometrium. The pharmacotherapies for the obesity treatment play an important role for increasing the weight loss established by diet and exercise alone, but actually, only a few antiobesity drugs are approved by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA). In particular, five medications have been approved in the USA for chronic weight management: orlistat, lorcaserin, phentermine/extended-release topiramate, naltrexone/bupropion, and liraglutide. Lorcaserin and phentermine/extended-release topiramate have not been approved in the European Union, in fact, several controversies and phentermine/extended-release topiramate have not been approved in the European Union, in fact, several controversies are still being debated and one of the major uncertainties, for patients with obesity, is whether current pharmacological treatments increase, reduce, or are neutral with respect to cardiovascular events. Moreover, drugs acting on the metabolism have considerable side effects and, for these reasons, pharmacological interventions for weight loss remain limited.

Several studies have provided evidence that carbonic anhydrase inhibitors (CAIs) are emerging as antiobesity drugs. To date 16 α-CA isoforms were reported in mammals with different tissue distribution and subcellular localization. These metallo-enzymes catalyze the reversible hydration of carbon dioxide to afford the ionic species bicarbonate and protons as follows: \( \text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+ \). The CA isoforms VA and VB, involved in lipogenesis, are expressed only in the mitochondria and the clinical observation of body weight reduction, for patients treated with the anticonvulsant topiramate and zonisamide (in phase II), is rationalized considering the potent inhibitory effect of these mitochondrial enzymes.

In this study, for the first time, essential oils (EOs) components were considered as a new class of potential CAIs. Traditionally, EOs have been largely employed as antibacterial, antifungal, and insecticidal agents, and several studies have confirmed these biological activities. In addition, EOs have been also considered for their analgesic, sedative, anti-inflammatory, spasmylytic, and locally anesthetic properties. Finally, EOs seem to have a great potential as anticancer therapeutic agents but mechanisms of action are still unknown, considering that EO extract is a very complex mixture of low weight molecules (20 to 70 components) at different concentrations in the phytocomplex.

Conversely, our study was focused on a specific interaction between the EOs content and the CAs family. Considering EOs as new resources of CAIs, in particular of the mitochondrial isoform VA, a structure-based virtual screening (SBVS) was performed in order to early identify in silico new potential antiobesity drugs. Therefore, the Essential Oil University (EOU) chemoinformatics database was used to build a chemical library of natural ligands that were virtually screened against several crystallographic X-ray structures of CA, deposited into the bioinformatics Protein Data Bank (PDB). The most relevant compounds selected on the basis of the predicted binding affinity results, due to the ligand-target interactions, were submitted to in vitro assays and new chemical scaffolds were discovered for CAIs.

**MATERIALS AND METHODS**

**In Silico Structure-Based Virtual Screening.** The 3D chemical structures of 2690 compounds, content in the EOU Web site, were downloaded and imported into the graphic interface of Maestro. In order to create the ligands database used for the SBVS, Duplicates were detected by the Canvas utility and Lipinski’s rule of five was used to filter 1771 compounds. For each molecule protonated and tautomeric forms at pH 7.4 were calculated, by the “LigPrep” module, ending up with a library of 2304 compounds. The lowest energy conformations, obtained by using the OPLS-2005 force field, represented the starting points for docking simulations. The X-ray crystallographic structures of CA I, CA II, and CA VA isoforms were selected from the PDB with the respective entry codes 1AZM, 4CQ0, and 1DMY, while for the CA VB enzyme no crystallographic models are deposited in the PDB. After the preparation procedure of each X-ray structure, the docking simulations were performed as reported in the experimental section of our previous SBVS study. Regarding the CA VA isoform, the best docked poses of compounds 1, 2, 6, and 11 were further submitted to a MD simulation of 100 ns, carried out by Desmond 3.7.43 Therefore, in order to...
analyze ligand-target interactions involved in the molecular recognition, we used the MD protocol already applied for investigating flavonoids inhibition of the monoamine oxidases isoforms. An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO$_2$ hydration activity. For compounds $k_{i}$ values were obtained from dose-response curves working at seven different concentrations of test compound, by fitting the curves using PRISM (www.graphpad.com) and nonlinear least-squares methods; values represent the mean of at least three different determinations as described by us previously. The inhibition constant ($K_i$) was then derived by using the Cheng-Prusoff equation as follows: $K_i = IC_{50}/(1 + [S]/K_m)$, where [S] represents the CO$_2$ concentration at which the measurement was carried out, and $K_m$ is the concentration of substrate at which the enzyme activity is at half-maximal. The concentrations of enzymes in the assay were as follows: hCA I: 11.7 nM, hCA II: 6.9 nM, hCA VA: 10.5 nM. The enzyme activity is at half-maximal. The concentrations of enzymes used in the assay were as follows: hCA I: 11.7 nM, hCA II: 6.9 nM, hCA VA: 10.5 nM. The enzyme is represented as a gray cartoon with transparent surface and all noncarbon atoms are colored according to atom types.

**RESULTS AND DISCUSSION**

Theoretical studies and in silico methods, by means of high performing computers, recently represent new innovative approaches in the drug discovery process. In fact, excluding the clinical trials, chemoinformatics and bioinformatics are playing important roles in every stage of the drug discovery pipeline. The EOU Web site is an example of a chemoinformatics database, providing accurate information about the world of EOs.

In this study, we have considered for the first time EOs as resources of antiobesity agents by inhibition of the mitochondrial CA VA/VB isoforms. With the aim of performing a SBVS, the 3D compounds used to build the ligands database were downloaded from the EOU Web site and prepared as reported in the experimental section. Regarding the X-ray crystal structures of the enzymes, we selected the PDB models of ubiquitous CA I, CA II, and mitochondrial CA VA, as previously reported for another SBVS study, performed in our laboratory for identifying new natural CAIs. Fortunately, in the PDB there are no crystal structures for the CA VB isoform and, therefore, it was not considered for the SBVS. Molecular docking simulations and the virtual screening procedure, already published, allowed us to select the new hit compounds on the basis of the theoretical affinity values, reported in Table 15.

Ten molecules, identified in silico as promising CA VA inhibitors, were submitted to in vitro assays using the human enzymes in order to confirm the ability to exert the predicted biological activity. In Table 1 for compounds 1–10 and the positive control acetazolamide, also called AZA (11), are reported inhibition constant ($K_i$) values, calculated as displayed in the experimental section. Biological results showed all compounds as weak inhibitors of the slow cytosolic isozyme CA I, demonstrated by $K_i$ values in the range of 3.44–8.81 μM, and ineffective inhibitors of the rapid cytosolic isozyme CA II with $K_i > 100$ μM, except for compounds 1, 3, 7, and 8. Regarding the mitochondrial CA VA, it was poorly inhibited by compounds 8 and 10, with $K_i$ values of 43.10 and 44.00 μM, while the other compounds showed a $K_i$ in the range of 3.80–9.69 μM. The 2-hydroxyisobutyric acid (2) proved to be the best CA VA inhibitor, with a $K_i$ value

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**Figure 1.** Best docked poses into the catalytic site of CA VA Xray model (PDB code 1DMY) with metal coordination of the zinc ion (Zn) and H-bond contacts for: acetazolamide 1 (a); cocrystallized inhibitor of the PDB model; and compounds 1 (d), 2 (b), and 6 (c). All ligands are rendered as cyan carbon sticks, THR199 amino-acid residue involved in the H-bond is shown as yellow carbon sticks, while histidines coordinating Zn sphere as yellow carbon lines. The enzyme is represented as a gray cartoon with transparent surface and all noncarbon atoms are colored according to atom types.
Table 1. 2D Chemical Structures of 10 Hit Compounds (1–10) Selected in Silico, by a SBVS Study, for the Inhibition Assays Against CA I, II, and VA Isoforms

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>TARGET</th>
<th>CAI</th>
<th>CAII</th>
<th>CA VA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>4.98</td>
<td>90.60</td>
<td>7.50</td>
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<tr>
<td>2</td>
<td></td>
<td>5.18</td>
<td>&gt;100</td>
<td>3.80</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>3.44</td>
<td>85.40</td>
<td>7.38</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>8.27</td>
<td>&gt;100</td>
<td>8.36</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>3.75</td>
<td>&gt;100</td>
<td>7.83</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>6.09</td>
<td>&gt;100</td>
<td>4.52</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>8.81</td>
<td>18.90</td>
<td>9.07</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>5.06</td>
<td>3.71</td>
<td>43.10</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>6.82</td>
<td>&gt;100</td>
<td>9.69</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>4.89</td>
<td>&gt;100</td>
<td>44.00</td>
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<tr>
<td>11</td>
<td></td>
<td>0.25</td>
<td>0.01</td>
<td>0.06</td>
</tr>
</tbody>
</table>

The traditional names of the new hit compounds are 1: β-thujaplicin; 2: 2-hydroxyisobutyric acid; 3: 4-isopropylbenzoic acid; 4: methyl geranate; 5: 3-phenylpropyl benzoate; 6: 3Z-nonenoic acid; 7: Z-geranic acid; 8: 2-methylhexanoic acid; 9: ferulic acid; 10: 5-methylfuraran-2-carboxylic acid. 11, Acetazolamide, is the CA inhibitor used as positive control. Biological data are reported as inhibition constant (K_i) values in μM.

equal to 3.80 μM, followed by 3Z-nonenoic acid (6) and β-thujaplicin (1) with K_i values 4.52 and 7.50 μM, respectively. Compound 9 has been already tested against several CA isoforms.

In order to analyze the ligand-target interactions, involved in the molecular recognition, and with the aim of taking into account the mechanism of action of new scaffolds, the best docked poses of compounds 1, 2, and 6, in complex with the CA VA X-ray structure 1DMY, were further investigated by a molecular dynamics (MD) simulation of 100 ns. Moreover, the reference 11 cocrystallized with the CA VA PDB model, was also used as positive control for the in silico simulations. In Figure 1, it is displayed a competitive mechanism of action for volatile compounds of EOs, coordinating the zinc ion and establishing other noncovalent ligand-target interactions with amino-acid residues of the CA VA catalytic site.

In particular, considering the different types of CAIs binding modes, all selected compounds showed metal-coordination binding to zinc ion (Figures 1 and 1S). Docking poses were submitted to molecular dynamics simulations (MDs) in order to rationalize the physical basis of the complexes, as reported in the plots of Figure 2S. For compound 2, the carboxyl group in the dissociated form and the hydroxyl substituent at C2 are key moieties in the metal coordination that was maintained for all MD simulation time (Figure 2).

Moreover, stabilization of the best pose is attributed to H-bond contacts with THR199 and ARG209, hydrophobic interactions with TRP209 and HIS94/96/119, also involved in ionic and π–π contacts (Figures 1, 1S, and 2S). The theoretical results showed a stabilization of compound 2, better than 1 and 6, but not better than AZA (11), due to the great number of H-bond contacts. These findings are summarized in Figure 3, as timeline representation of H-bond contacts established between enzyme and ligand over the course of the MD trajectory.

Enzyme activity analysis is only a preliminary investigation but it is the first step, in the drug discovery process, for identifying new potential antiobesity agents targeting the CA V isoforms.

Actually, there are several patents with mitochondrial CAIs, also in combination therapy, confirming that inhibition of enzymes involved in de novo lipogenesis represents a new valid approach for the obesity treatment. In addition, from a polypharmacological prospective, EOs contents and their derivatives may be considered multi target ligands (MuTaLig), in fact, other experimental results reported in literature have shown EOs compounds as potential histone deacetylase inhibitors (HDACIs), promising targets in the cancer therapy, by binding the zinc ion also present in this metallo-enzyme.

In conclusion, in this article, docking and MDs enabled us to investigate the binding modes of ligands against the CA VA target, confirming the important role of molecular modeling techniques to speed up the identification of bioactive compounds, reducing time and cost of research procedures and taking into account multi target interactions. Moreover, EOs and other natural products could be investigated in silico in order to early identify new potent antiobesity drugs and/or new scaffolds to focus the lead optimization studies, considering that it has been demonstrated that CA VA inhibition with sulfonamides changes the metabolism of pyruvate, acetate, and succinate in mitochondria and reduces dramatically lipogenesis.

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World Health Organization; EMA, European Medicines

innovative ligand identification

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Table 1S: theoretical affinity results of molecular docking
for compounds 1–11. Figure 1S: 2D structures of 1, 2, 6, and
11 with ligand-target interactions of the best docked
poses. These contacts were also monitored during MDs and reported in Figure 2S (PDF)

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the
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Notes

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ABBREVIATIONS USED

EOs, essential oils; CAIs, carbonic anhydrase inhibitors; WHO, World Health Organization; EMA, European Medicines

Agency; FDA, Food and Drug Administration; SBVS, structure-based virtual screening; EOU, Essential Oil University;
PDB, Protein Data Bank; AZA, acetazolamide; $K_i$, inhibition constant; MD, molecular dynamics; MDs, molecular
dynamics simulations; hCA, human carbonic anhydrase; MuTaLig, multi-target ligands; HDACIs, histone deacetylase

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Figure 3. Timeline representation of H-bond contacts that CA VA target makes with ligands 1 (green), 2 (red), and 6 (yellow) compared to AZA 11 (blue) over the course of the MD simulations.
(33) The Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB): http://www.rcsb.org.

