Sawa Medical Journal

Reprint from

Vol.1 Issue.2 2025

Sawa Medical Journal

Anti cancer potential of green biocatalysts (Si@OSA-Dir.Si@SA) using carbonic anhydrase

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Abstract

Carbonic anhydrase (CA) is an important biological target in cancer therapy, due to its vital role in regulating intracellular pH and providing an acidic environment that promotes the growth and spread of cancer cells. Tumor cells rely on aerobic glycolysis, which leads to the accumulation of acidic products such as CO_2 and lactic acid, thus lowering the pH in tumor tissue. In this study, molecular docking demonstrated that SA and OSA possess a high binding capacity to the active site of CA II, with SA recording the highest binding energy (-8.13 kcal/mol). Hydrogen bonding was documented within the active pocket, supporting the stability of the enzyme complex. CA was purified from red blood cells using systematic steps that included hypotonic lysis, hemoglobin removal using chloroform and ethanol, and precipitation with ammonium sulfate.

Subsequently, the effectiveness of nanocatalysts (Si@SA and Si@OSA-Dir) at different concentrations (up to 500 µg/mL) was evaluated, demonstrating their ability to raise the pH of the medium and reduce acidity. The Si@OSA-Dir catalyst demonstrated significant effectiveness in stabilizing pH at 8 within 20 minutes, while the other compounds reached pH 7.7, demonstrating their effectiveness in adjusting acidity.The results indicate that these prepared compounds hold promise as CA inhibitors and have potential applications in cancer treatment strategies by targeting the acid balance in the tumor microenvironment.

Keywords: Carbonic anhydrase (CA)، pH regulation، Cancer microenvironment، Enzyme inhibition، Enzyme purification، Antitumor activity.

Introduction

It has recently been demonstrated that the pH differential in tumor cells is produced and maintained by the carbonic anhydrase family [1]. Over the past 20 years, research has shown that CAs are essential for tumor cell survival as well as for their metabolism, migration, and invasion [2]. This has led a number of groups to try to figure out how to target CAs in order to stop the spread of cancer. It is generally acknowledged that CAs, just like in normal tissue, are the primary driver of pH regulation in primary tumor cells [1]. Through their activity, these enzymes control the synthesis of bicarbonate, the all-purpose physiological buffer [3]. Reversible biochemical pathways share this process. The balance between acidity and alkalinity (pH) in biological systems is impacted by the activity of CA, which is controlled by specific compounds [4].

The catalytic activity of the enzyme can be slowed down or stopped entirely by these substances, which are referred to as CA inhibitors. These substances can affect the hydration and dehydration of carbon dioxide (CO_2), a reaction essential to preserving pH balance, by blocking CA [5]. By attaching itself to the enzyme's active site, an inhibitor can stop it from interacting with CO_2 and H_2O. The rate of pH change may be slowed down or stopped entirely by this disturbance.

Alkalization therapy is being investigated as a possible adjunct to traditional cancer treatments. Its goal is to neutralize the acidic tumor environment in cancer. This entails employing alkalizing agents to raise the tumor's pH, which is normally acidic because of the metabolism of cancer cells [6]. Because cancer cells frequently use a lot of glucose, the environment around tumors becomes acidic. Because of this acidity, cancer may become more aggressive, spread to other body parts, and worsen the prognosis for patients[7]. Changes in certain cell functions may be caused by or result from the acidity imbalance. Cancers typically have higher levels of H^+ production and excretion[8].

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

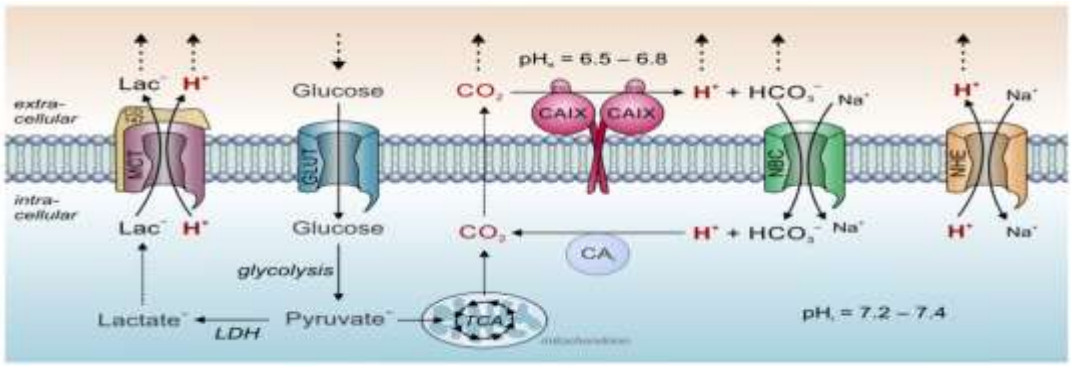


Figure 1 : Tumor pH regulation by carbonic anhydrase and acid/base transporters. In tumor cells, metabolic acids are produced primarily by glycolysis and subsequent hydrolysis of ATP (lactate− + H+), and mitochondrial respiration CO_2[8].

Experimental

2.1 Raw materials

All chemicals were used directly without further purification. Ethanol (100%) was purchased from GCC, chloroform (99.4%) from Scharlau, and ammonium sulfate (99.5%) from Fisher, BP212R-1.

2.2 Samples characterizations

Enzyme identification was performed using FTIR technology.

2.3.Enzyme extraction

The blood samples were obtained from donors. The samples were placed in a vial with an anticoagulant centrifuged at 4700 rpm for 10 min at 4°C. The compacted red cells (erythrocytes) were washed three times with saline (0.9% NaCl) and then lysed with deionized water at 4 °C followed by vigorous agitation. The plasma membrane was removed by filtration.

2.3.1.Extraction of carbonic anhydrase

Chloroform and ethanol were used for the extraction and purification of the enzyme. The suspensions were stirred and centrifuged to remove excess chloroform, and the supernatant was filtered to remove any remaining precipitate [9]. The isolated enzyme purified by ammonium sulphate precipitation. Solid $(NH_4)_2SO_4$ was added gradually to obtain protein solution. Precipitated purified enzyme was separated by centrifugation at 5000 rpm for 20 min.

2.3.2.Anticancer carbonic anhydrase inhibitors:

Human carbonic anhydrase isoforms which are tumor-associated, overexpressed enzymes in hypoxic tumors, being involved in metabolism, pH regulation, ferroptosis, and overall tumor progression. A new method was proposed in this study to confirm the anticancer activity of the compounds by affecting the enzyme's pH.

2.3.3. Enzyme activity as an anti-cancer

An enzyme solution was prepared by taking 0.2 mg of the purified enzyme and dissolving it in 50 mL of deionized water. Concentrations of the test sample were then prepared at 500 µg/mL. One mL of the enzyme was taken, and 100µL of test sample was added to it, and inhibitory studies were measured by employing catalyst as substrate against the human CA extracted .The ability of the prepared catalysts to inhibit or block the recombinant enzyme catalytic function was investigated. The influence of (time and conc.) on carbonic anhydrase activity have been studied as follows.

2.3.3.1. Effect of incubation time on carbonic anhydrase activity

The reaction mixture consisting of enzyme and test sample was incubated at 20, 40, 60, and 80 minutes in a shaker water bath, then the enzyme activity was measured to determine the optimal time for incubation [10] .

2.3.3.2. Concentration effect of carbonic anhydrase activity

The optimal test sample concentration that supports maximal carbonic anhydrase activity was evaluated using different concentrations of test sample (100 µg/mL,250 µg/mL and 500 µg/mL), and carbonic anhydrase activity was measured.

Result and dissection

3.1.Swiss Target Prediction

It was thought that the molecules (NH_2-SO_3H and NH_2-O-SO_3H) which are immobilized on silica could undergo hydrolysis and be released under physiological conditions, as results, the potential bioactivity of these two molecules were predicted using in silico approach named "Ligand-Based virtual screening", this approach allowed us to predict the most potential macromolecular targets of the used molecules, the prediction was achieved using the online tool "Swiss Target Prediction". The prediction is founded on the similarity with a library of 370,000 known bioactive compounds on more than 3000 proteins.

The results showed a short list of six potential targets all of them are Carbonic anhydrases as illustrated in Table 1.

Table 1: Virtual screening results of Compounds SA and OSA.

Molecule	Target	Common name	Target Class
	Carbonic anhydrase I	CA2	Lyase
NH_2-SO_3H	Carbonic anhydrase II	CA7	Lyase
	Carbonic anhydrase IV	CA1	Lyase
	Carbonic anhydrase VI	CA6	Lyase
	Carbonic anhydrase VII	CA9	Lyase
	Carbonic anhydrase IX	CA4	Lyase
NH_2-O-SO_3H	Carbonic anhydrase I	CA1	Lyase
	Carbonic anhydrase II	CA2	Lyase

3.1.1.Molecular docking simulation

Although, the two molecules (SA and OSA) revealed potential activity towards several isozymes (Carbonic anhydrases), however Carbonic anhydrase II was highlighted due to its abundance in Red Blood Cells, and, since the protocol involves extraction of this enzyme from human red blood cells, this enzyme (pdb: 1a42) was subjected to molecular docking for validation of the virtual screening results and to predict the mechanisms of action. The active site of CA-II was identified by Domsic et al. [11], where the following amino acids: Gly63, His64, His94, His96, His119, Val121, Val143, Leu198, Thr199, Thr200, Val207, and Trp209, were involved in the active site. Based on the obtained results, compound SA and OSA, showed a slight difference in the binding affinity, the highest binding affinity

was recorded for compound SA (-8.13 kcal/mol), while the binding energy of compound S2 was -7.87 kcal/mol. The protein-compound complex was visualized using the tool PLIP, the results showed that both compounds (SA and OSA) positioned inside the active site forming hydrogen bonds (2 and 1, for SA, and OSA, respectively) as illustrated in Figure 2.

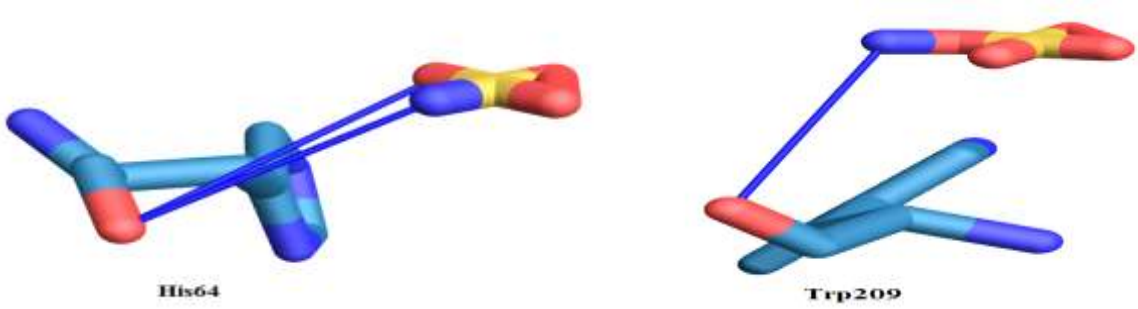


Figure 2: Binding modes of compounds SA and OSA inside the active site of carbonic anhydrase II (pdb: 1a42).

3.2.Carbonic anhydrase

3.2.1.Purification of CA

The process described is a method for purifying a target enzyme from red blood cells by inducing red blood cell lysis to release components, then selectively removing it and other cellular components before precipitating the desired protein using ammonium sulfate. The red blood cells are first suspended in an isotonic 0.9% saline solution, then placed in a hypotonic deionized water solution to trigger hemolysis and cell lysis, followed by filtration to remove the cell membranes. Hemoglobin is then removed through precipitation and separation with chloroform and ethanol. Finally, the target protein (enzyme)is precipitated multiple times using ammonium sulfate at highest concentrations (60% to 80%) being used in a high-salt environment to selectively precipitate the target protein. This entire process, including the ammonium sulfate precipitation, is repeated multiple times to increase the purity of the extracted enzyme.

3.2.2. FT-IR spectral analysis of extracted enzyme

FTIR analysis confirmed the successful extraction and structural integrity of the CA enzyme by identifying key functional groups in the extracted sample (Figure 3.(b)) and matching them to a standard spectrum (Figure 3.(a)). The extracted enzyme showed peaks corresponding to O-H and N-H stretches around 3400-3300 cm^{-1}, C-H aliphatic groups near 2900 cm^{-1}, C=O (carboxylic) at 1650-1670 cm^{-1}, and C-O/C-N bonds of amino acid side chains around 1080-1004 cm^{-1}. [12]. The presence of these specific functional group peaks confirmed the successful extraction of the CA enzyme and demonstrated that its structure remained intact, as evidenced by the strong correlation between the extracted sample's spectrum and the standard enzyme spectrum.

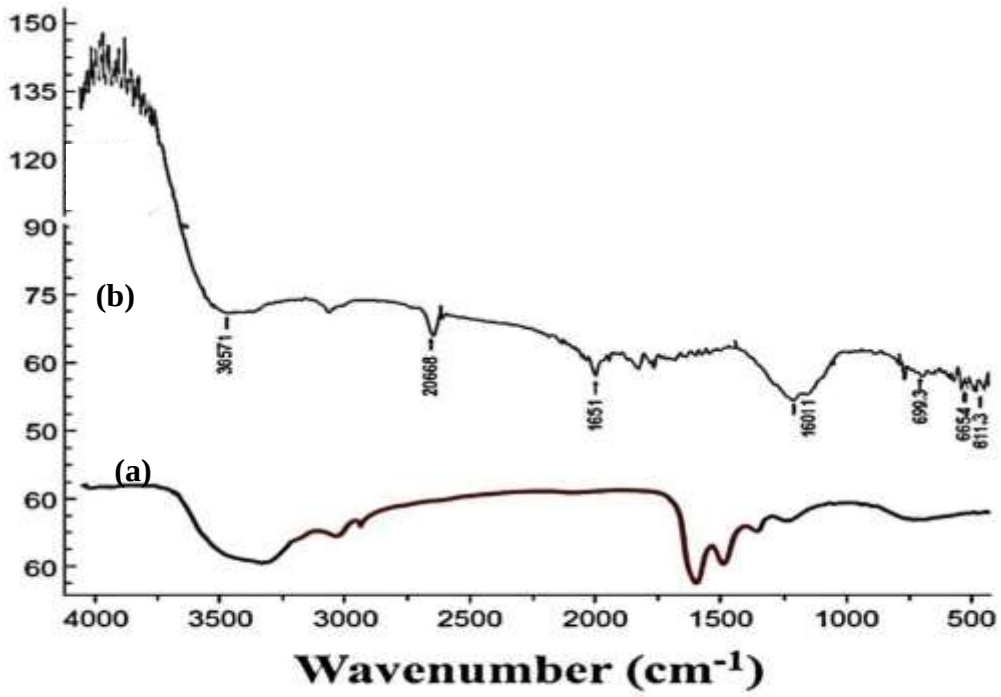


Figure 3: shows two (FT-IR) spectra, where spectrum (a) is a standard for comparison[13] and spectrum (b) shows the extracted carbonic anhydrase.

3.2.3.Enzyme activity as an anti-cancer

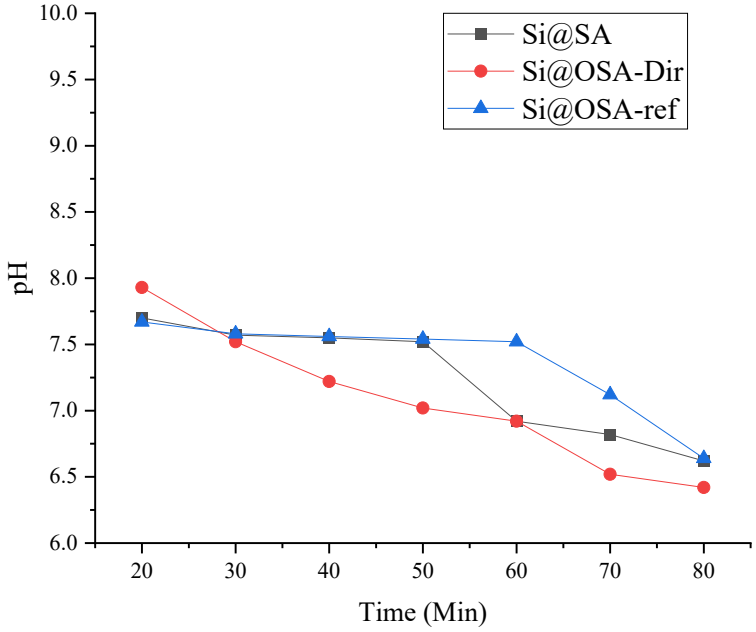
CA is an enzyme that plays a crucial role in regulating pH within human cells and, in the context of cancer, is implicated in the development of a tumor microenvironment that favors cancer cell growth and metastasis. The acidic tumor pH is an attractive target because it is a common phenotype that is found across a wide spectrum of cancers. Numerous studies on cancer metabolism over the past decade have revealed that cancer cells exhibit aerobic glycolysis due to the activation of oncogenes, although these in vivo studies suggest that CO_2 will be a significant source of metabolic acid production in cancer cells, the higher pK of carbonic acid (pKa 6.35) means that CO_2 would have a much smaller acidifying effect than lactic acid. In normal, healthy individuals, the pH measurement was 6.63. Subsequently, tests were conducted by adding the substance under study at different concentrations. Figure 5. shows the magnitude of the pH change. The most effective concentration for the enzyme's pH was 500µg/mL, which lowered the enzyme's acidic pH and made the medium more balanced. The results provided insight into the nature of the substances and their ability to lower the enzyme's acidity. These results confirm the effectiveness of these substances as anticancer agents.

3.2.4. optimal condition for CA in the presence of catalysts

3.2.4.1.Effect of incubation time on carbonic CA

To find out how well these biocatalysts inhibited CA, they were used once more after being synthesized and characterized in this study. Interfering with pH regulation is a promising approach for

the development of anti-cancer drugs because pH is frequently dysregulated in tumors [14]. Figures 4 and 5 show the inhibitory effects of these catalysts. The enzyme treated with catalyst Si@OSA Dir reached the stable pH (pH: 8) in 20 minutes, whereas Si@OSA ref and Si@SA both had stable pH values of 7.7 Figure 4. These results indicate that the prepared catalysts are capable of cleaning the acidic end products due to their shifted pH.



Figuer 4: Effect of time on the pH of carbonic anhydrase enzyme treated with the studied biocatalysts.

3.2.4.2. Effect of Catalyst Concentration on CAs

Comparable pH growth curves were also obtained with different concentrations of new biocatalysts, as the CA enzyme is essential for the progression of cancer because it regulates pH and promotes tumor cell survival and metastasis [14]. At varying concentrations (100 µg/mL to 500 µg/mL), the tested biocatalysts were found to exhibit inhibitory activities against CA. When compared to the absolute pH under normal conditions (6.6 prior to enzyme treatment with prepared catalysts), Si@SA demonstrated a greater increase in pH than Si@OSA ref and Si@OSA-Dir, respectively Figuer.5. The findings show that these catalysts have the ability to raise pH by accepting protons (H^+) or donating hydroxide ions (OH-). This is explained by the presence of SO_3H hydroxide ions on the surface of catalyst particles, which combine with those of o-sulphonic and sulphamic acid to form hydrogen bonds, raising the pH of the solution and turning it alkaline. Lastly, more investigation is necessary to confirm that these pH regulators are viable targets for chemoprevention and cancer treatment.

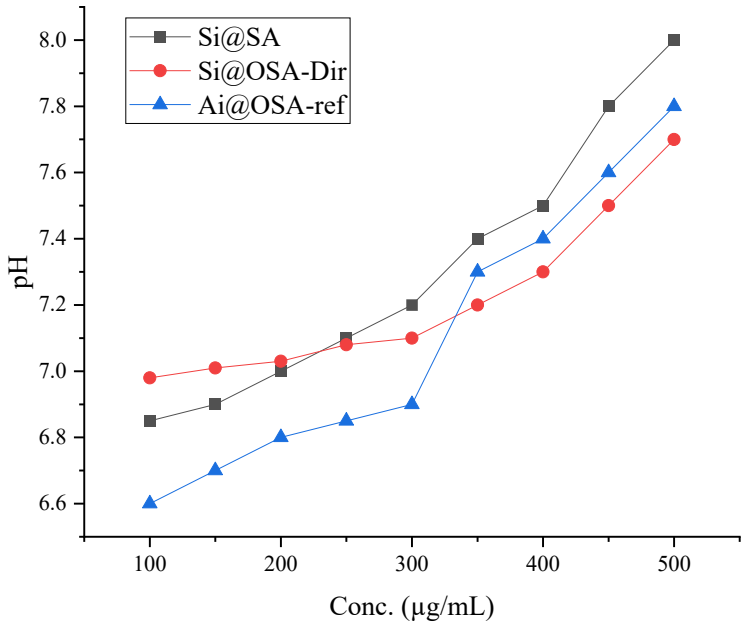


Figure 5: Effect of different synthesized catalysts in various concentrations on pH of carbonic anhydrase extracted from human RBCs.

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