Drug Design, Development and Therapy

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ORIGINAL RESEARCH

Citric Acid Controls the Activity of YopH Bacterial Tyrosine Phosphatase

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Purpose: Citric acid (CA) is a tricarboxylic acid with antioxidant and antimicrobial properties. Based on previous studies, the small compound with its three carboxylic groups can be considered a protein tyrosine phosphatase inhibitor. YopH, a protein tyrosine phosphatase, is an essential virulence factor in *Yersinia* bacteria.

Materials and Methods: We performed enzymatic activity assays of YopH phosphatase after treatment with citric acid in comparison with the inhibitory compound trimesic acid, which has a similar structure. We also measured the cytotoxicity of these compounds in Jurkat T E6.1 and macrophage J774.2 cell lines. We performed molecular docking analysis of the binding of citric acid molecules to YopH phosphatase.

Results: Citric acid and trimesic acid reversibly reduced the activity of YopH enzyme and decreased the viability of Jurkat and macrophage cell lines. Importantly, these two compounds showed greater inhibitory properties against bacterial YopH activity than against human CD45 phosphatase activity. Molecular docking simulations confirmed that citric acid could bind to YopH phosphatase.

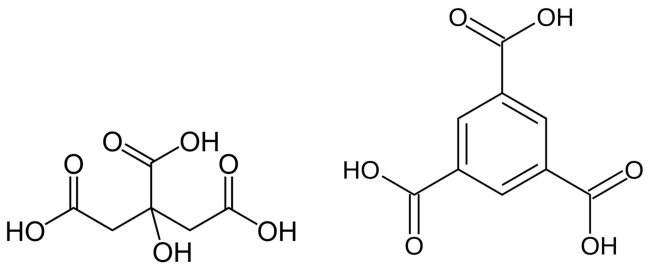
Conclusion: Citric acid, a known antioxidant, can be considered an inhibitor of bacterial phosphatases.

Keywords: protein tyrosine phosphatase, citric acid, trimesic acid, bacterial virulence factors, YopH, *Yersinia*, CD45 phosphatase

Introduction

Citric acid is a tricarboxylic acid with six carbons (Figure 1), which was first crystallized from lemon juice. It is a basic natural acid in citrus fruits and is found, for example, in limes, lemons, tomatoes, strawberries, strawberries, and cranberries. Interestingly, its production may also occur in the fungi *Aspergillus niger* and the yeast *Yarrowia lipolytica*.[1]

Citric acid has been utilized as a preservative, but also has many other properties such as antioxidant, acidifying, and flavoring.[2,3] It has been established across many industries in the form of food, beverages, drugs, and beautifying products. Citric acid is highly soluble in water and quickly degrades through membranes.[3]



Citric acid
$C_6H_8O_7$

Trimesic acid
$C_9H_6O_6$

Figure 1 Citric acid and trimesic acid formulas.

Received: 5 December 2023
Accepted: 27 February 2024
Published: 11 April 2024

Citric acid has been shown to exhibit superior antiviral effect. This causes irreversible damage to the virus. Accordingly, citric acid may be used for permanent disinfection of contaminated surfaces.[4]

Citric acid possesses antimicrobial properties against bacteria transmitted through food, eg *Escherichia coli, Salmonella, Listeria*.[5] *Yersinia enterocolitica* is a bacterium that causes infections after consumption of undercooked meat products, unpasteurized milk, or water contaminated with bacteria.[6]

The secretion of virulence factors is a characteristic phenomenon exploited by some virulent bacteria, such as *Yersinia* or *Salmonella*. Virulence effectors injected into host cells change signaling pathways and initiate the infection process. These virulence factors include bacterial protein tyrosine phosphatases, the activities of which are essential for bacterial pathogenicity. *Yersinia* spp. use a type III secretion model for the translocation of virulence effectors, such as YopH phosphatase. The protein tyrosine phosphatase YopH is involved in inhibiting phagocytosis by macrophages to eliminate pathogens.[7]

Translocation of the bacterial YopH factor into phagocytic cell types inhibits the immune response of the infected host. YopH activates the disruption of focal adhesions[8,9] and inhibits phagocytosis.[10,11] This causes the release of tumor necrosis factors and oxidants.[12,13] YopH also impairs T-lymphocyte and B-lymphocyte function[14] at very early stages and prevents an adaptive inflammatory response crucial for the survival of bacteria in the lymph nodes of the host.[15]

Previously, we reported that compounds possessing carboxylic acid groups in their structure may be considered potential inhibitors of protein tyrosine phosphatases. A compound with a structure similar to the most effective inhibitors known to date is aurintricarboxylic acid.[16,17]

In this study, we analyzed the effect of citric acid in comparison to trimesic acid, which is also a promising inhibitory compound because of its small size and the presence of three carboxylic groups, similar to citric acid and aurintricarboxylic acid. We performed inhibitory assays of YopH bacterial phosphatase in comparison to one of the human phosphatase CD45 presented in hematopoietic cells. The enzymatic activity of phosphatase was measured to determine whether citric acid and trimesic acid could decrease the activity of YopH phosphatase. We also measured the cytotoxicity of citric and trimesic acids in Jurkat T E6.1 and macrophage J774.2 cell lines. We performed molecular docking analysis of the binding of citric acid molecules to YopH phosphatase to analyze the ability of citric acid molecules to bind and interact with YopH phosphatase.

To achieve the objectives of this study, we employ a rigorous and systematic approach. This involves conducting a comprehensive literature review to identify gaps in current knowledge and highlight the significance of further investigation. Additionally, we employ appropriate research methodologies, such as docking, inhibitory activity and cytotoxicity analysis, to collect and analyze data. By utilizing these methods, we aim to obtain reliable and valid results that can inform evidence-based decision-making.

Materials and Methods

Reagents

YopH and CD45 phosphatases were obtained from Merck (Darmstadt, Germany). Cell lines were purchased from The European Collection of Cell Cultures (ECACC). Citric acid, trimesic acid, cell media, supplements, and other reagents were obtained from Sigma–Aldrich.

Cell Culture

We performed cell culture experiments with JurkatT E6.1 and J744.2 cell lines. The JurkatT E6.1 cells are human blood cells (leukemic T-cell lymphoblast). J744.2 is mouse macrophage-like cell line.

The cells were cultured in RPMI medium supplemented with 10% fetal bovine serum, 100 µg/mL penicillin/streptomycin, and 2 mM L-glutamine. Cultures were maintained at 37 °C in an atmosphere containing 5% CO_2. The cell culture density was maintained at a maximum of 1×10^6 cells/mL. At least every two days the medium was replaced with fresh medium, and the cells were counted and reseeded to maintain the recommended density.

Cell Viability/Cytotoxicity Test

The MTT test used enables the measurement of the activity of mitochondrial enzymes in the cell, which is directly proportional to the amount of reduced tetrazolium salt, and thus may be an indicator of cell viability.

The cells (1×10^6 cells/mL) were either untreated (control) or treated with various concentrations of citric acid and trimesic acid solutions, and after the appropriate incubation time were suspended in a 5 mg/mL MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) in RPMI without phenol red. Then, 100 µL samples were incubated for 3–4 h at 37 °C in 96-well plates. When the purple precipitate was clearly visible under the microscope, 100 µL DMSO was added to each well and the covered plate was left in the dark for 15 min. Absorbance was measured at 590 nm using a microplate reader.

YopH Enzymatic Activity Assay

The solution of the recombinant YopH and CD45 phosphatases was prepared in 10 mM HEPES buffer, pH 7.4. The final concentration of phosphatase in the reaction samples was 1.5 µg/mL. Enzyme samples were either untreated (control) or treated with various concentrations of citric acid and trimesic acid solutions. The assay was performed in 96-well microplates and the final volume of each sample was 200 µL. The enzymatic activity of phosphatases was measured at 37 °C and 405 nm on a microplate reader (Jupiter; Biogenet) using DigiRead Communication Software (Asys Hitech GmbH) with 2 mM *para*-nitrophenyl phosphate (*p*NPP) chromogenic substrate.

Phosphatase Activity Assay in Cell Lysates

Cells were either untreated (control) or treated with citric acid and trimesic acid solutions. After incubation, the cells were lysed and protein tyrosine phosphatase activity was assessed. The assay was performed in 96-well microplates and the final volume of each sample was 200 µL. The enzymatic activity of phosphatases was measured at 37 °C and 405 nm on a microplate reader (Jupiter; Biogenet) using DigiRead Communication Software (Asys Hitech GmbH) with 2 mM *para*-nitrophenyl phosphate (*p*NPP) chromogenic substrate. The activity of total protein tyrosine phosphatases in the cell lysates was assessed by measuring the amount of total protein using the Bradford method.

Docking Studies

The initial structure of YopH was imported from the RCSB Protein Data Bank (www.pdb.org) using code 2YDU.pdb.[18] The structure was minimized using the taff.ff force field of Molecular Operating Environment software (MOE; Chemical Computing Group). Chain A of this PDB file contained 306 residues. The ligand was removed from the PDB file, and citric acid was docked into the structure of YopH. A docking simulation was performed, in which the grid box was assumed to be the center of the protein. The simulation was performed using AutoDock Vina Software.[19]

Statistical Analysis

All experiments were performed at least five times. Data were analyzed using GraphPad Prism (GraphPad Software, v.4, La Jolla, CA, USA). Statistical analyses were performed using ANOVA combined with Tukey's test or a *t*-test combined with the Wilcoxon test. Data are expressed as the mean ± SD. Differences between means were considered statistically significant at $p<0.05$.

Results

Citric Acid and Trimesic Acid Decrease the Activity of Recombinant YopH Phosphatase

To determine whether citric acid and trimesic acid could decrease the activity of YopH tyrosine phosphatase, we performed YopH activity assays. We studied the inhibitory effects of citric acid and trimesic acid at different concentrations. The effects of selected concentrations of citric acid and trimesic acid are shown in Figure 2.

Both citric acid and trimesic acid are able to reduce the activity of YopH phosphatase at micromolar concentrations and are also important at nanomolar concentrations. Such low concentrations of citric acid and trimesic acid could not acidify the buffer. Both compounds induced YopH inactivation in a reversible manner. YopH activity was restored after treatment with dithiothreitol (DTT), a thiol reducer.

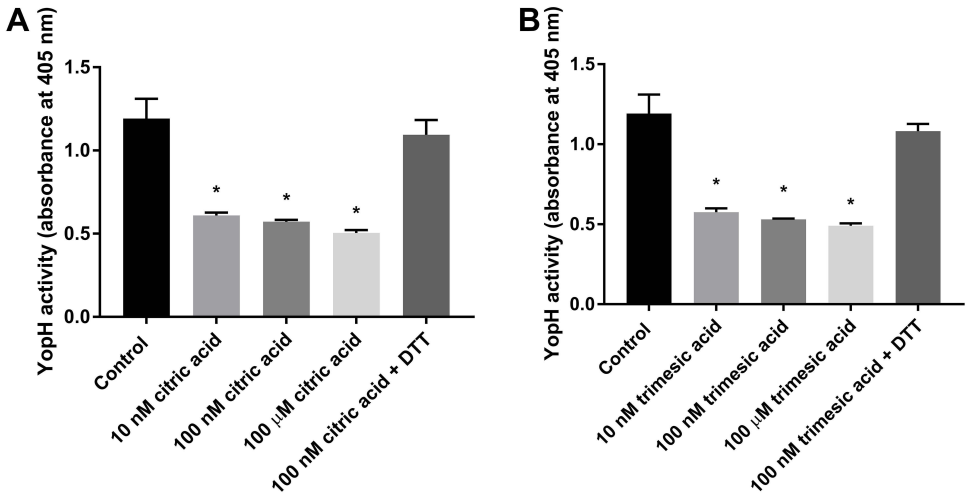


Figure 2 The YopH recombinant enzyme activity after treatment with: (**A**) citric acid; (**B**) trimesic acid. Control is the activity of YopH treated only with buffer. Statistical analysis was performed with one-way ANOVA test, *P<0.0001.

Citric Acid and Trimesic Acid are More Potent Inhibitors of YopH Than CD45 Phosphatase

We measured the inhibitory effect of citric acid and trimesic acid on the activity of bacterial YopH phosphatase in comparison to that of human CD45 phosphatase, another protein tyrosine phosphatase. We chose CD45 phosphatase because it is an abundant enzyme in Jurkat cells and is present in macrophages, which are both targets of bacterial virulence factors.

The IC_{50} values for each compound were calculated. We calculated IC_{50} values based on a plot presenting citric acid concentration versus percentage of the enzymatic activity of recombinant YopH measured as absorbance with *p*NPP substrate. The *p*NPP concentration for IC_{50} calculations was 2 mM equal to Km value determined for YopH, where Km value is defined as substrate concentration at which enzyme activity is at half maximal. The results are presented in Table 1.

Importantly, citric acid and trimesic acid were more effective at inhibiting YopH phosphatase activity than CD45 phosphatase activity. Thus, we conclude they are more specific inhibitors of the bacterial YopH factor than human phosphatase CD45.

The results indicate that citric acid and trimesic acid are able to inhibit 50% of YopH activity in the concentration range of 105–116 nM, while inactivating CD45 requires concentrations of 603 nM and 505 nM, respectively (Table 1). According to these data, citric acid is six times more effective as an inhibitor of bacterial YopH than human CD45 phosphatase.

Citric Acid and Trimesic Acid Negatively Affect the Viability of Macrophages and Jurkat T Cells

As the activity of the virulence factor YopH is concentrated on disrupting hematopoietic cell pathways, we decided to measure the impact of citric acid and trimesic acid on macrophages and T-cell lines. We compared the impact of citric acid and trimesic acid using nanomolar working concentrations of these compounds to avoid acidification of the medium.

Table 1 The Comparison of IC_{50} Values

Phosphatase	Citric acid	Trimesic acid
YopH	116 nM	105 nM
CD45	603 nM	505 nM

We performed MTT test to assess the viability of the cells. The MTT test enables the measurement of the activity of mitochondrial enzymes in the cell, which is directly proportional to the amount of reduced tetrazolium salt, and thus may be an indicator of cell viability.

We reported that citric acid and trimesic acid had negative effects on the viability of macrophages (Figure 3). Stronger effects of these compounds were observed on the viability of Jurkat T cells (Figure 4). Citric acid and trimesic acid significantly reduced the viability of Jurkat T cells.

Total Protein Tyrosine Phosphatases Activity in Cell Lysates of Jurkat and Macrophage Cell Lines

Next, we investigated whether the treatment of macrophages and T-cell lines with citric acid and trimesic acid would have an impact on total phosphatase activity within these cells. To assess these effects, we performed protein tyrosine

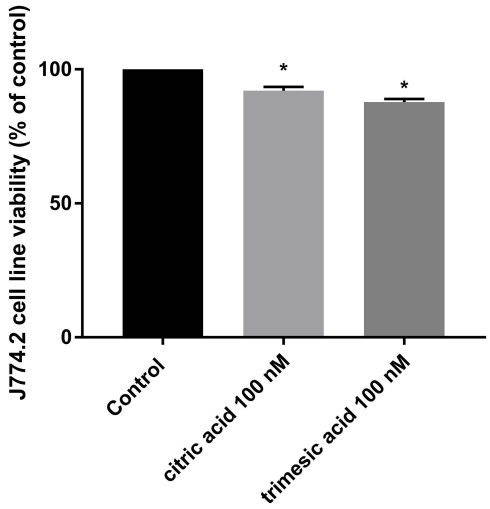


Figure 3 The viability of macrophage cell line J774.2 after treatment with 100 nM citric acid and trimesic acid. Control is the viability of cells treated only with medium. Statistical analysis was performed with one-way ANOVA test, *P<0.0001.

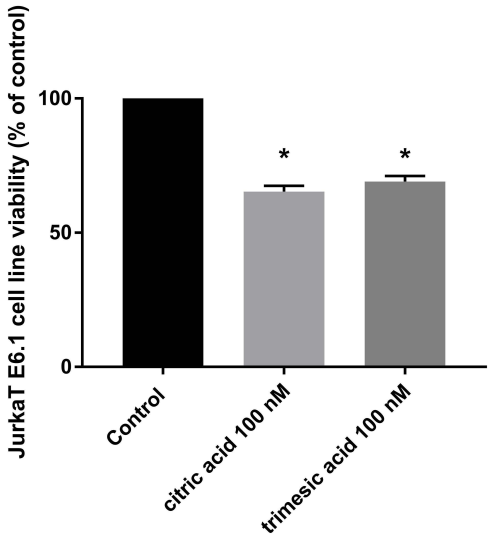


Figure 4 The viability of Jurkat T cell line E6.1 after treatment with: 100 nM citric acid and trimesic acid. Control is the viability of cells treated only with medium. Statistical analysis was performed with one-way ANOVA test, *P<0.0001.

phosphatases activity assays using cell lysates obtained from macrophages and Jurkat T cells. The cells were treated with citric acid and trimesic acid, lysed, and the total protein tyrosine phosphatases activity measured in the cell lysates.

The results indicated that the total protein tyrosine phosphatases activity in Jurkat T cells was not reduced by citric acid treatment (Figure 5). In contrast, the phosphatase activity of macrophages was reduced by citric acid treatment (Figure 5). The same effect was observed for trimesic acid (Figure 6).

These results may be explained by the fact that the phosphatome of Jurkat T cells mainly consisted of CD45 phosphatase, which is less susceptible to inactivation by citric acid and trimesic acid.

Docking Analysis of Citric Acid Binding to YopH Phosphatase

Finally, using molecular docking, we analyzed the ability of citric acid molecules to bind and interact with YopH phosphatase. Docking simulations were performed using Auto Dock Vina software [ref.docking]. Based on the calculated binding affinities, we obtained the nine best binding simulations, of which the four best binding positions are shown in Figure 7. The binding affinity for the best binding pose was calculated as −5.5 kcal/mol, and the values for the selected binding poses are presented in Table 2.

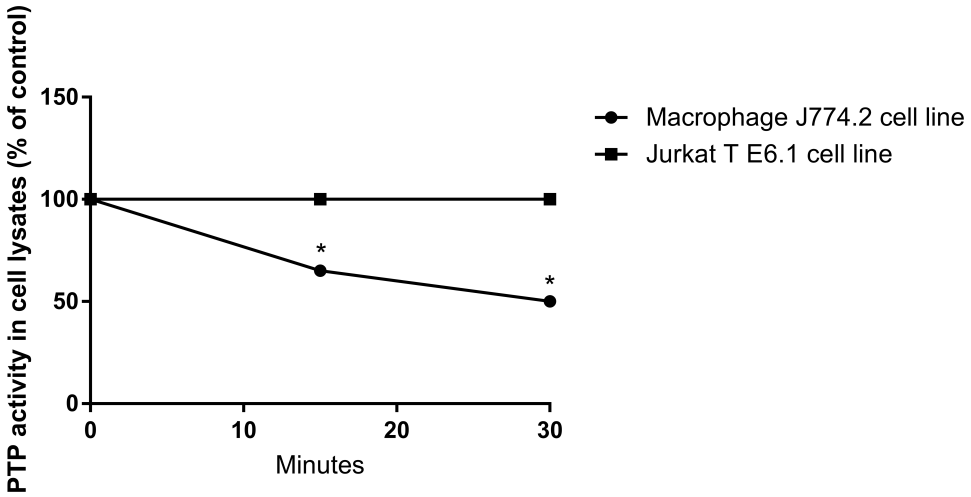


Figure 5 The total protein tyrosine phosphatases activity in cell lysates after treatment with 100 nM citric acid. Control is the protein tyrosine phosphatases activity of cell lysates treated only with buffer. Statistical analysis was performed with one-way ANOVA test, *P<0.0001.

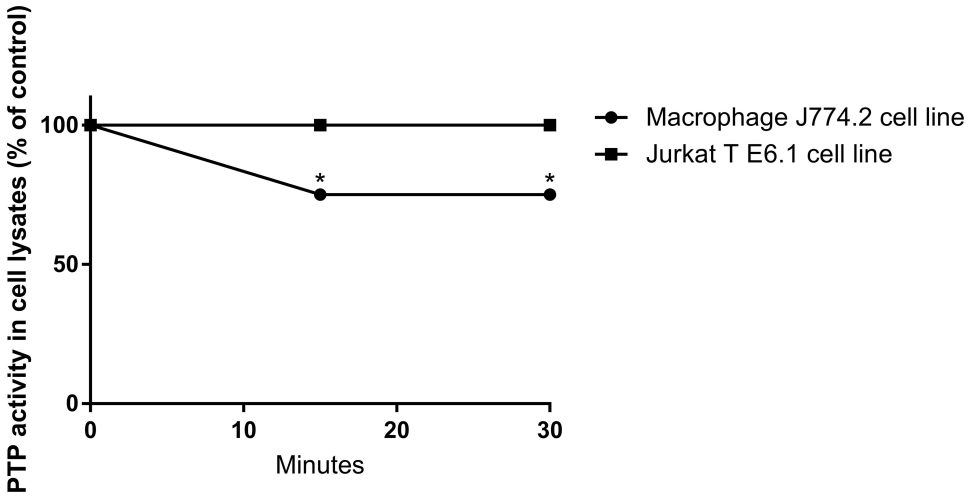


Figure 6 The total protein tyrosine phosphatases activity in cell lysates after treatment with 100 nM trimesic acid. Control is the protein tyrosine phosphatases activity of cell lysates treated only with buffer. Statistical analysis was performed with one-way ANOVA test, *P<0.0001.

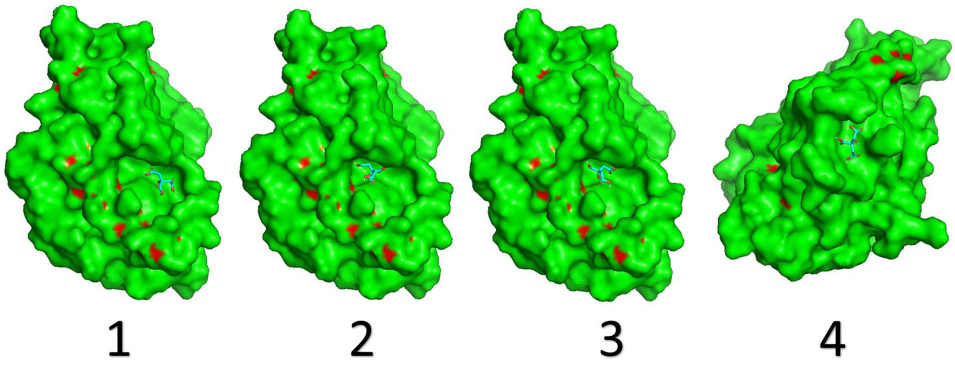


Figure 7 The best four (1–4) predicted binding simulations of citric acid compound to YopH phosphatase binding sites. The best poses were selected from docking analysis based on the top score of binding affinities (see Table 2).

We focused on the best predicted binding pose with the best binding affinity (mode 1 form Figure 7) and searched for possible interactions. We studied the binding sites connected to amino acid residues and the area surrounding them. As shown in Figure 8, the citric acid molecule was not predicted to bind to the catalytic center of YopH phosphatase, but the binding site was a short distance from the active site. Amino acid residues such as Arg398, Arg255, Asp394, and Leu397 are probably involved in binding of citric acid molecules. In close proximity, amino acid residues such as Cys403 and Arg404, which form the catalytic pocket of the enzyme, are essential for enzymatic activity.

Discussion

During infection, *Yersinia* bacteria utilize the virulence effector YopH to dephosphorylate host proteins. The YopH effector plays a role in the bacteria's capacity to defend against peritoneal macrophage phagocytosis in host cells. YopH protein is a tyrosine phosphatase[20,21] with a catalytic domain at the C-terminus that is structurally related to eukaryotic protein tyrosine phosphatases.[22] It is extremely difficult to create selective inhibitors of all tyrosine phosphatases because of their shared characteristics. A Pro-rich region and a multifunctional N-terminal domain that binds to tyrosine-phosphorylated target proteins follow after the catalytic domain.[23,24]

Several proteins have been identified as substrates of YopH in various cell types. In epithelial cells, the three potential substrates are p130Crk-associated substrate, paxilin or the focal adhesion kinase (FAK). In macrophages, the substrates are p130Cas, Fyb (Fyn binding protein),[25] SKAP-HOM,[26] and Pyk (a tyrosine kinase homologous to FAK). There are also YopH substrates located in T cells, such as Lck, LAT, and SLP-76.[27,28] Most of these proteins are tyrosine kinases or

Table 2 The Binding Affinity Values for the Best Nine Selected Binding Modes of Citric Acid to YopH Phosphatase Calculated Using AutoDock Vina Software

mode	Affinity [kcal/mol]	Distance from the Best Mode	
		rmsd l.b.	rmsd u.b.
1	−5.5	0.000	0.000
2	−5.4	4.159	6.549
3	−5.3	4.133	6.273
4	−5.2	28.890	30.339
5	−5.1	26.428	28.297
6	−5.0	29.457	30.521
7	−5.0	0.953	4.109
8	−5.0	27.107	28.139
9	−5.0	1.238	4.200

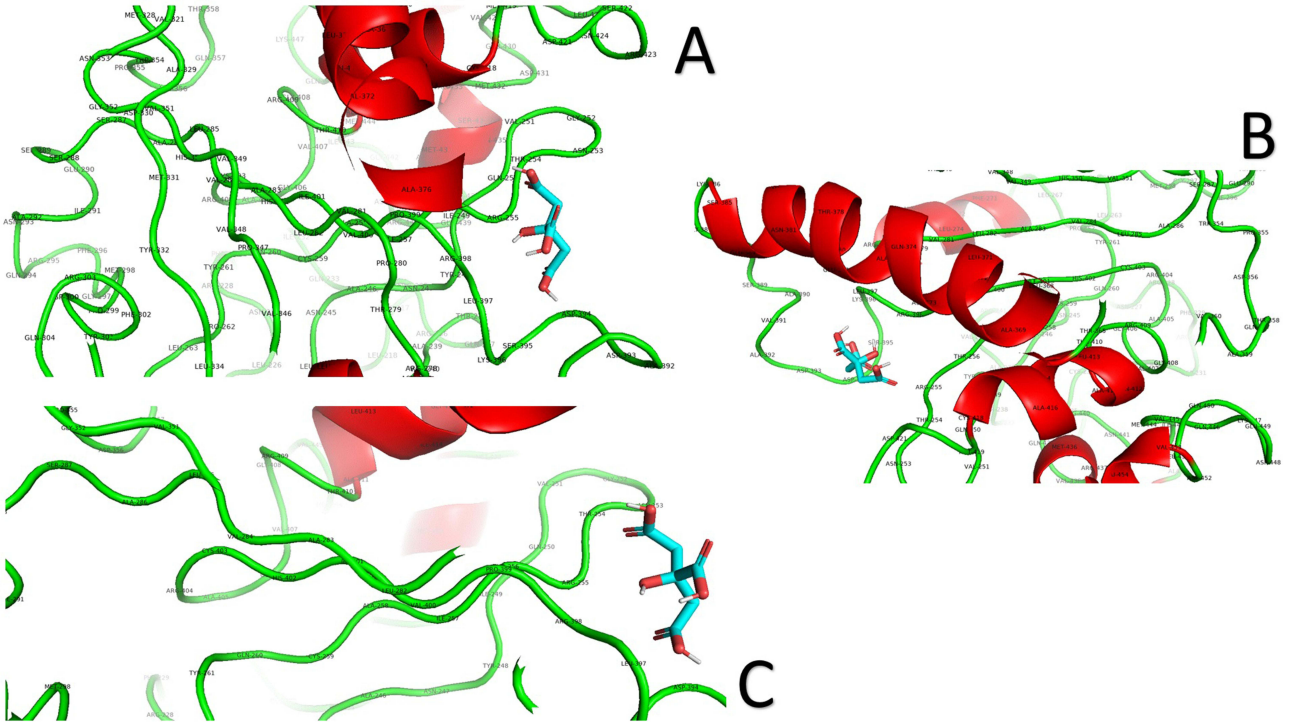


Figure 8 Amino acid residues and surroundings of the best predicted binding pose of YopH phosphatase with citric acid. The best binding pose was visualized from three different angles (**A**–**C**). The best binding pose is based on the top score of binding affinity.

adaptors. All of these substrates are implicated in phagocytosis-involved pathways or activation of the early stages of signal transduction in the immune response of hematopoietic cells.[15]

Here, we have shown the inhibitory effect of citric acid and trimesic acid treatment on the activity of bacterial YopH phosphatase. In comparison we calculated IC_{50} values of each compound against human CD45 phosphatase in hematopoietic cells. Interestingly, the results indicate that citric acid and trimesic acid are more specific inhibitors of bacterial YopH than of human CD45 phosphatase.

Weak organic acids have been used for centuries to treat infections. Recently, there has been a resurgence of interest in the use of weak acids for treatment of bacterial infections.[29] There are several potential methods for the action of weak acids in bacteria. The main method involves lipophilic characteristics enabling the uncharged form of weak acids to diffuse freely across the bacterial cell membrane and into the cytoplasm.[30] Weak acids also cause a decrease in intracellular pH, which is responsible for bacterial growth reduction.[29] It is assumed that the destabilization of bacterial membrane function caused by weak acids is the primary reason for their antimicrobial properties.[31] Other studies have also indicated that weak acids cause anions to accumulate inside the cytoplasm, which induces an osmotic effect and changes metabolic processes within bacterial cells.[29]

Citric acid is a weak tricarboxylic acid with three pKa values. Citric acid at a concentration of 40% has been shown to prevent re-colonization of biofilms and reduce the survival rate of *Pseudomonas* bacteria in biofilms.[32,33] Here, we have shown that citric acid, even at nanomolar concentrations, can induce inactivation of bacterial YopH phosphatase.

Citric acid can be considered a suitable protein tyrosine phosphatases inhibitor, as it is a small compound and was proved by computational analysis to bind to phosphatases. According to previous studies, the presence of carboxylic groups can explain the inhibitory potential of citric acid and trimesic acid. Similar to the strongest known inhibitors of YopH phosphatase, aurintricarboxylic acid, both citric acid and trimesic acid contain three carboxylic groups.[16]

Conclusion

This study showed that citric acid, a known antioxidant and natural fruit compound, has inhibitory properties against the bacterial virulence factor YopH, which is responsible for blocking the phagocytosis process of macrophages during infection.

One of the great advantages of citric acid compounds is undeniably the fact that they are not harmful to humans, as well as being non-toxic to the environment and very effective.[3] The need to exploit naturally originating antimicrobials is growing in importance.[34] Moreover, bacteria are gaining resistance to utilized chemical antibiotics over time, and there is a need to use compounds with broader effects.[5] This study would be more valuable if the *Yersinia* bacterial infection model was included. However, only a few laboratories worldwide have access to and permission for *Yersinia* strains and studies on bacterial models are extremely limited. Here, we present studies including the recombinant YopH model from *E. coli* bacteria, but further studies using other bacterial strains that utilize protein tyrosine phosphatases as virulence factors (eg, *Staphylococcus aureus*) are underway.

Acknowledgments

M.G.-P. acknowledges ST46 (Medical University of Gdansk, Poland).

Funding

This research was funded by IDUB Program's Research Project for Young Scientists MTN No. 71-1215 (Medical University of Gdansk, Poland).

Disclosure

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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