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Original Research

The inhibition of *Candida albicans* secreted aspartyl proteinase by triangular gold nanoparticles

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Abstract

Objective(s): The aim of this study was to synthesize triangular gold nanoparticles, and then to evaluate their capability for inhibition of *Candida albicans* secreted aspartyl proteinase 2 (Sap2).
Materials and Methods: To synthesize the nanoparticles, hydrogen tetrachloroaurate and hexadecyl trimethyl ammonium bromide were incubated in presence of Sn(IV) meso-tetra(N-methyl-4-pyridyl) porphine tetratosylate chloride, and then characterized. Next, thirty clinical isolates of *Candida albicans* were obtained from patients suffering from vaginal candidiasis. Each *Candida albicans* isolate was first cultured in YCB-BSA medium, incubated for 24 h at 35 ºC. Then, 100 µL of triangular gold nanoparticles at three concentrations (16, 32, and 64 µg/mL) were added to *Candida* suspension, and incubated for 24 and 48 h at 35 ºC. To evaluate Sap activity, 0.1 mL of medium and 0.4 mL of 0.1 M sodium citrate buffer (pH 3.2) containing BSA 1% w/v were added, and incubated 15 minutes at 37 ºC. Then, the optical density of each tube was read at 280 nm. Enzyme activity was expressed as the amount (µM) of tyrosine equivalents released per min per ml of culture supernatant.
Results: This study showed that the size of the nanoparticles was 70±50 nm. Sap activity evaluation demonstrated triangular gold nanoparticles could inhibit the enzyme, and the higher incubation time and concentration led to more decrease of Sap activity.
Conclusion: For the first time, we demonstrated triangular gold nanoparticles as a novel inhibitor of Sap enzyme which may be useful for treatment of candidiasis.

Keywords: *Candida albicans*, Peptide ligand, Secreted aspartyl proteinase, Triangular gold nanoparticles

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Introduction

Extracellular hydrolytic enzymes are important elements for pathogenicity of most important fungi, e.g. Trichophyton mentagrophytes (1), *Aspergillus fumigatus* (2), *Cryptococcus neoformans* (3), and *Candida albicans* (4). Of the hydrolytic enzymes, secreted aspartyl proteinases (Sap), phospholipase B enzymes, and lipases are the most important enzymes produced by *Candida albicans* (4-6). Moreover, Sap proteinases are the key virulence factor of *Candida albicans* (7, 8). They are encoded by 10 genes, and Sap2 is the major gene which encodes a pre-pro-protein, 35,880 Da (9). Other Candida species including *Candida dubliniensis* (10), Candida tropicalis (11), and *Candida parapsilosis* express Sap genes (12).

Antibody can inactivate Sap enzymes (13) which opens up a new window to treat *Candida albicans* infections. Another inhibitor is pepstatin A, an enzyme which can digest mucosal proteins (14). On the other hand, peptidomimetic inhibitors can inactivate Sap enzymes of *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida lusitaniae* (15). Moreover, some renin inhibitors such as A-70450 and A-79912 can also inhibit Sap enzymes. Also, A-70450 and A-79912 inhibit Sap2 activity in vitro, but they are ineffective to inhibit in vivo (16).

Metal nanoparticles such as silver and gold have antifungal activity, and may be able to inactivate Sap enzyme. But it must be noted the activity of metal nanoparticles is shape-related. For example, shape-related antimicrobial activity has been observed for gold nanoparticles (17). It was found that spherical gold nanoparticles were generally more toxic than rod-shaped nanoparticles (17). Also, spherical gold nanoparticles had good biocompatibility, but rod gold nanoparticles were more toxic than cube gold nanoparticles (18). Although there are few data on antimicrobial property of different shapes of silver and gold nanoparticles in literatures (17-20), but no data were found about Sap inactivation of and gold nanoparticles. The aim of this study was to investigate the capability of triangular gold nanoparticles to inhibit *Candida albicans* Sap2 enzyme.

Material and Methods

Materials

Hydrogen tetrachloroaurate ($HAuCl_4$), triethanolamine (TEA), hexadecyl trimethyl ammonium bromide (CTAB), Sn(IV) meso-tetra(N-methyl-4-pyridyl) porphine tetra-tosylate chloride (SntMepyP), bovine serum albumin (BSA), pepstatin A, and trichloro-acetic acid (TCA) were purchased from Sigma-Aldrich chemical company, USA. Sabouraud dextrose agar (SDA), Sabouraud dextrose broth (SDB), and yeast carbon base (YCB) medium were purchased from Invitrogen, UK. Ammonia and sodium citrate buffer were provided from Zyst Fannaver Shargh Company, Iran.

Synthesis and characterization of triangular gold nanoparticles

10 mL of 20 mM $HAuCl_4$ were added to 50 mL of 0.2 M TEA/0.1 M ammonia. Then, 100 mL of 10 mM CTAB, 50 mL of 3mM SntMepyP, and 1000 mL of water were added, and irradiated for one hour by a discharge lamp (ZFS Company, Iran). To characterize triangular gold nanoparticles, transmission electron microscopy (TEM) (Hitachi, Japan), UV-VIS spectrophotometer (Novin gostar Company, Iran), and dynamic light scattering (DLS) (ZFS Company, Iran), Fourier transform infrared spectroscopy (FTIR) were used (21).

Preparation of Candida albicans isolates

Thirty clinical isolates of *Candida albicans* were obtained from patients suffering from vaginal candidiasis. These patients were from different health center of Yazd and Tehran, Iran. It must be noted that an informed consent was obtained from each patient. The isolates were

previously confirmed by CHROMagar (BD Diagnos-tics, USA) and RFLP-PCR at Shahid Beheshti University of Medical Sciences, Tehran, Iran. All isolates were first inoculated on SDA, incubated for 48 h at 37 ºC, and then one colony of each isolate was immersed to 10 mL of SDB. After 24 hours incubation at 37 ºC, *Candida* cells were harvested by centrifugation (1500 g, 10 min), washed with phosphate buffered saline, and 10 mL of RPMI1640 medium was added to reach to 10^5 cells/mL.

Evaluation of Sap2 activity

Each *Candida albicans* isolate was first cultured in YCB-BSA medium, incubated for 24 h at 35 ºC in the 24-wells plate. After incubation, the cells were washed with normal saline twice by centrifugation at 5000 rpm for 5 min. Then, 106 cells inoculated into 25 mL of YCB-BSA medium (22). In the next step, 100 µL of triangular gold nanoparticles at three concentrations (16, 32, and 64 µg/mL) were added to *Candida* suspension, and incubated for 24 and 48 h at 35 ºC. After incubation, 0.1 mL of medium was added to 0.4 mL of 0.1 M sodium citrate buffer (pH 3.2) containing BSA 1% w/v, and incubated 15 min at 37 ºC. Then, 0.5 ml of 5% TCA was added to stop reaction. The mixture was centrifuged at 3000 g for 10 min, and the OD of each tube was read at 280 nm against distilled water. Enzyme activity was expressed as the amount (µM) of tyrosine equivalents released per min per ml of culture supernatant (22). In the negative control tube, 0.1 ml of 50 µg/mL pepstatin A was used instead of culture supernatant.

Statistical analysis

All tests were carried out three times, and the results were shown as the mean ± standard deviation (SD).

Student's t-test was used, to detect significant difference by SPSS software (version 16.0 for Windows; SPSS Inc., USA), and P. value <0.05 was regarded as statistically significant.

Results

Characterization of triangular gold nanoparticles

UV-VIS spectrum, DLS graph, and TEM image are shown in the Figure 1a, 1b, and 1c, respectively. Figure 1a shows that triangular gold nanoparticles have two sharp peaks at 540 nm and 750 nm. Figure 1b demonstrates the size distribution of triangular gold nanoparticles. As seen, their mean ± standard deviation was approxi-mately 70±50 nm. Their polydispersity index (PdI) was 0.12.

Triangular shape of the gold nanoparticles was confirmed by TEM (Figure 1c). Based on FTIR peaks (Figure 1d), triangular gold nanoparticles have sharp bands on the 600, 1100, 1300, and 1600 cm ($^{-1}$).

The Sap2 activity results

Figure 2a and Figure 2b show the Sap activity when *Candida* cells incubated with three concentrations of triangular gold nanoparticles for 48 and 24 hours, respectively. This study showed that the higher concentration led to more decrease of Sap activity. Also, the higher incubation time led to more decrease of Sap activity. The lowest Sap activity was near 4 µM for concentration of 64 µg/ml and incubation time of 48 hours.

This result was obtained from thirty isolates. In the both incubation times, significant differences were observed between the uptake of triangular gold nanoparticles at concentration of 16 µg/mL vs. 32 µg/mL and 64 µg/mL (by t-test, P<0.05). The Sap activity of negative control was 20±2 µM.

Discussion

Although Sap enzyme is a key virulence factor of Candida albicans, it can be inactivated by different biomolecules, e.g.

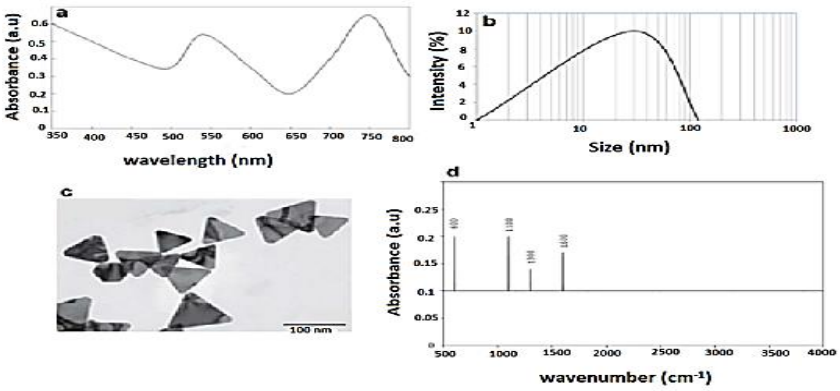

Figure 1. Characterization of triangular gold nanoparticles. UV-VIS spectrum (a), DLS graph (b), TEM image of triangular gold nanoparticles (c), FTIR peaks (d) of triangular gold nanoparticles at concentration of 32 µg/mL. For better interpretation, we omitted the noise signals, and only main peaks were shown.

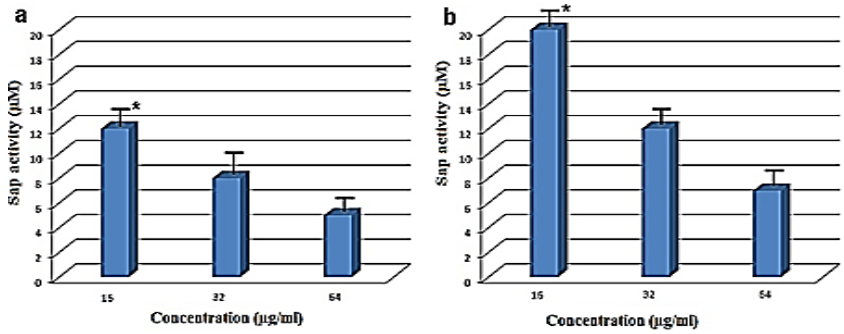

Figure 2. The Sap activity when *Candida* isolates incubated with conjugated triangular gold nanoparticles, peptide ligands, and triangular gold nanoparticles alone after 48 hours (a) and 24 hours (b). * P<0.05 compared with the Sap activity of triangular gold nanoparticles at 64 µg/mL and 32 µg/mL.

antibody (13), pepstatin (14), peptide-mimetic inhibitors (15), and renin inhibitors (16). As seen in the Figure 3 (15), the active site of Sap enzyme is very narrow, about 2 nm. Here, we wanted to investigate the inhibition of *Candida albicans* Sap2 enzyme by triangular gold nanoparticles. At the first step, triangular gold nano-particles were synthesized and character-rized. The synthesis protocol was according to Miranda article (21) with some modifications. In the method, $HAuCl_4$ was reduced by CTAB and TEA, and capped by SntMepyP. We changed only final concentration of reactant. After synthesis, two sharp peaks at 540 nm and 750 nm were detected by UV-VIS spectroscopy. Also, DLS showed the size of nanoparticles was approximately 70±50 nm, with PdI= 0.12. In our previous study, we worked on the antifungal properties of different shapes of gold and silver nanoparticles (23). We evaluated nanocubes, nanospheres, and nanowires,

and it was found that silver and gold nanocubes had the highest antifungal activity against *Candida albicans*, *Candida glabrata* and *Candida tropicalis*. For the first time, Smitha et al showed high antimicrobial activity of triangular gold nanoparticles against *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus niger*, and *Fusarium oxysporum* (24).

This study showed that triangular gold nanoparticles inhibit Sap2. The inhibition pattern of the nanoparticles was dose and time dependent. The lowest Sap activity was near 4 µM for concentration of 64 µg/ml and incubation time of 48 h.

The design of Sap inhibitors has been previously done by Cele Abad-Zapatero at Abbott Laboratories (25). They found that Sap2 activity could be inhibited by some renin inhibitors such as A-70450 and A-

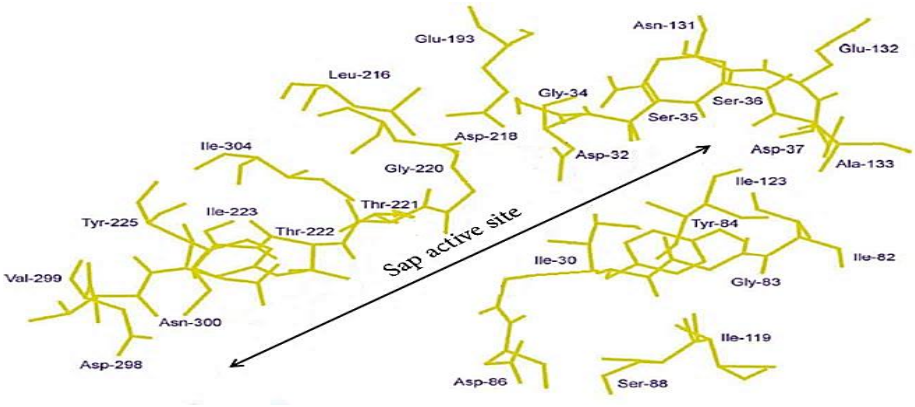

Figure 3. The schematic representation of Sap2 active site (15).

79912. Also, Cadicamo et al designed and synthesized three libraries of inhibitors, based on the structure of pepstatin A with some changes at the P3, P2, and P2′ position. They showed that these inhibitors had high inhibitory potencies against *Candida albicans* Sap1, Sap3, Sap5 and Sap6 (26). Importantly, triangular gold nanoparticles may have several problems when used in vivo, including the lack of protection, inhibitor potency, and specificity. It must be mentioned all of restrictions may be existed for our work, and must be checked in the future study.

Conclusion

In conclusion, we presented triangular gold nanoparticles as a new inhibitor of Candida albicans Sap2 enzyme. It was found that triangular gold nanoparticles could inhibit Sap2 with dose and time dependent manner, compared with control.

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