Exploration of Mechanistic Insights of Acemetacin in Melanogenesis Through Zebrafish Model, Enzyme Kinetics, Molecular Docking and Simulation Approaches

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Abstract

The present study describes the anti melanogenesis effect of Acemetacin (ACE). Essential protein (melanin) that is vital for the skin for defense from UV rays. In the present research, emerging drug ACE was examined for its melanin inhibition using three different (in vitro, in vivo and computational) methods. ACE showed remarkable potency ($IC_{50} = 0.353 \pm 0.003 \mu M$) against tyrosinase in the comparison of standard, kojic acid ($IC_{50} = 16.841 \pm 1.161 \mu M$) and ACE exhibited competitive inhibition. In the *in vivo* study zebrafish embryos were exposed with 5, 10, 15 and 20 μM of ACE and same doses for positive control (Kojic Acid). At 72 h treatment, ACE expressively ($P < 0.001$) reduced the level of pigmentation (62.89%) at a concentration of 20 μM , relative to that of kojic acid (39.64%). The binding profile of ACE was confirmed by molecular docking and the stability of the docked complexes was justified by MD simulation. Based on our results, it was concluded that ACE possessed good therapeutic potential against melanogenesis by targeting the tyrosinase.

Keywords: Acemetacin, Tyrosinase inhibition, Melanogenesis, Computational studies, Zebrafish

Introduction

Nowadays, globally, the investment in skin bleaching mediator is approximately increase by up to US\$ twenty-three billion by 2020. Asia is observed to be the biggest market for skin whitening agents, mainly India, China, Japan and Korea [1]. Human skin, hair and eyes are dependent on the creation of melanin and its amount, worth and dissemination. Melanin shows an instrumental part in safeguarding the skin in contradiction to dangerous outcomes of UV radiations and oxidative trauma from different ecological contaminants [2]. The natural pigment melanin is synthesized from skin layers' cell

melanocytes via the process of melanogenesis [3, 4]. Embryonic neural crests are the stem cells of melanoblasts, these unpigmented cells have further proceeded towards pigmented melanocytes [5, 6]. In the epidermis of the skin, every melanocyte is enclosed by about 36 keratinocytes which are high in the ratio [7].

Melanin plays out an essential part in shielding the human skin however the anomalous union and high measure of melanin-delivering cells in various particular organs of the humans and animals lead to many harmful and dangerous

skin diseases, for example, melisma, melanoderma after inflammation, freckles, and wrinkles. Furthermore, exposure to UV-radiation can cause damage in DNA, mutations in genes, development of cancer and destruction of the immune system [8].

Melanosomes, the lysosome like organelles, are present in the melanocyte. These organelles produce two types of melanin pigment, one is black-brown or insoluble dark polymer known as eumelanin present in dim skin and dark hair, and the other is yellow to red and soluble, that is present in freckle skin type and red hair [9].

Melanogenesis is a very specialized process and involves multiple sequences of enzymatic and chemical processes. In these pathways, three enzymes, namely tyrosinase, dopachrome tautomerase and tyrosinase-related protein-1, are very significant intermediates of melanogenesis. In specific, tyrosinase is fully essential for the synthesis of melanin. The production of melanin started with the conversion of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) or dopaquinone. L-DOPA acts as the substrate in the case of eumelanin and pheomelanin production [10, 11].

The natural decarboxylation of dopachrome produces 5,6-dihydroxyindole which quickly oxidizes and jointly produces insoluble black to brown pigment known as 5,6-dihydroxyindole-melanin. Conversely, if tyrosinase-related protein 1 is present, dopachrome conversely, in the presence of tyrosinase-related protein 1, dopachrome will produce dihydroxyindone-2-carboxylic acid (DHICA), which then oxidize and polymerize to form DHICA-melanin. This type of pigment is slightly soluble and medium in size and lighter brown in color. In short, the presence of melanin activating catalysts and substrates determines the types of pigment synthesized. Tyrosinase assumes a significant job in speed controlling all the while of oxidation because other whole mechanism proceeds with optimum pH [12].

The embryo of zebrafish is a very common vertebrate animal model for physiological and biochemical studies because of

high similarity with mammals, hence the embryo of zebrafish has been developed as a model for animal experiments [13]. Zebrafish has various benefits, such as the production of large amounts of embryos, easy care of embryos, smaller size, and most importantly high efficacy of penetration of drugs through skin and gills. In the past, several literature reports have shown that most of the *in-vivo* phenotype-based experiments have been performed on zebrafish [14-16].

Acemetacin (ACE) is a glycolic acid ester of Indometacin, structurally. ACE is a non-steroidal compound with the potency of treating inflammation used for the treatment of osteoporosis, osteopenia, severe gout, toothache, dysmenorrhea, lower back pain and relieving post-operative pain [17, 18].

Although some indole containing compounds have been reported as tyrosinase inhibitors [19], yet, as literature search evident that there are no significant findings available for ACE as melanogenesis inhibitor. Therefore, considering the pharmacological importance of ACE, in the present investigation, we have explored its tyrosinase inhibitory potential using three approaches; *in vitro* enzyme inhibition, computational docking and dynamic simulation, and *in vivo* zebrafish model for determination of pigment inhibition potency.

Materials and Methods

The analytical grade chemicals and reagents for all experiments were purchased from Sigma-Aldrich Co. Korea.

Mushroom tyrosinase inhibition assay

The potency of ACE against tyrosinase was investigated by exactly following our published method in various articles [19-21]. In the first step, 20 mM of phosphate buffer at pH 6.8 was prepared, and 140 μ L of the solution was transferred in each well of the assay plate. Simultaneously, 20 μ L of mushroom tyrosinase (target enzyme) was also added from 30 U/mL of stock solution. In a third step, 20 μ L of ACE was transferred into the assay plate. After the first incubation of 10 min at 25 $^{\circ}$ C, the 20 μ L of

substrate L_DOPA was added from the 0.85 mM stock solution, and the reaction mixture was incubated for another 20 min at room temperature. Finally, the change in absorbance was recorded at wavelength 475 nm on SpectraMax ABS microplate reader. For comparison and assay validation, kojic acid was used as a positive control and assay buffer was used as a negative control. For IC₅₀ calculations, all concentration was investigated separately and repeated for three time in order to obtain better accuracy of results. For calculation of IC₅₀ through nonlinear regression GraphPad Prism was used. By following bellow mentioned equation % inhibition was determined.

$$\text{Tyo sin ase \% inhibition} = \left[\frac{\text{Bank} - \text{Sample}}{\text{Bank}} \right] \times 100$$

Kinetic analysis

The kinetic experiment was carried to reveal the behavior of ACE in tyrosinase inhibition. Range of doses was applied for the identification of the pattern of inhibition of tyrosinase by ACE via kinetic evaluation using our published methods [22, 23]. In total, three doses of ACE were investigated, i.e. 0.00, 0.353 and 0.706 μM . Various serious of L-DOPA doses were used from 0.0625 to 2 mM for all experiments. The first incubation and reading period was the same as presented in the above inhibition method for IC₅₀ calculations. The maximum first velocity was determined using the primary linear phase of absorbance for 5 min after mixing the enzyme solution at thirty seconds period. The enzyme blockage pattern was determined using the Lineweaver Burk graph of the opposite of velocities (1/V) against the opposite of used substrate doses. Another chart was drawn for calculations of the enzyme inhibition dissociation constant K_i through 1/V against the ACE doses.

Determination of pigmentation reducing capacity of ACE in embryos of Zebrafish (In Vivo)

The animal experiments were carried out as accordance with the published methods [21, 24].

Zebrafish farming

The selected animal zebrafishes were obtained from the local market and maintained in our fish facility laboratory for a period of thirty days. All conditions were optimized for fish culture as prescribed in literature for zebrafish maintains. Shrimp larvae were used as the food and fishes were housed in tanks made up of thermostatic material. For respiration, growth and better health, air and water filtration were maintained. The fish seed was obtained using the normal procedure of fish spawning performed using the light source as a stimulator. All animal experiments methods were confirmed by Departmental review Board of Kongju National University (IRB NO. 2011-2).

ACE treatment and depigmentation examination

Firstly, the E3 medium was prepared by mixing 5 mM sodium chloride, 0.17 mM potassium chloride, 0.33 mM calcium chloride and 0.33 mM magnesium chloride. Then, the fish embryos were obtained using pipette into an assay plate three to four embryos were transferred in each well. Subsequently, the ACE solution was prepared in 0.1% DMSO added into the embryos medium for nine to seventy-two h post-fertilization. For comparison and assay confirmation, kojic acid was used as positive control. Then, chorion of embryos was removed and tricaine methanesulfonate MS-222 was used as anesthesia. Lastly, embryos slide was prepared using 1% methylcellulose on the whole slide and images were taken using stereomicroscope purchased from Nikon, Japan.

Quantification of melanin (In Vivo)

Melanin pigment quantification assay was carried out by published methods [21,25,26]. For quantification of melanin and dose selection trails were performed and a 20 μM dose was chosen for animal experimentations. The obtained fingerling fishes were placed in dishes with the help of dropper, where thirty-three embryos were used in the plate and dosage with ACE and kojic acid, final volume of each well, adjusted with assay E3 medium as 2 mL. After the drug exposure time, anesthetized embryos were cleaned with E3

medium and their eyes were removed from all used embryos and the crude extract was prepared using centrifugation and homogenization methods. The crude pellet of the extract was mixed in 1 mL of sodium hydroxide and heated at 100 °C for ten min. The quantity of melanin was calculated based on the absorbance at 405 nm and the findings were determined with the curve of purchased melanin from sigma. Whole experimental methods were repeated thrice.

Statistical analysis

Values were expressed as the mean $\pm$ the standard error of the mean. The (t-test) was performed for the experiments and overall data showed significance results with the value of $P < 0.001$.

Computational Methodology

Retrieval of mushroom tyrosinase

For mushroom tyrosinase 3D structure, we used a freely available database of protein structures where 2Y9X PDB ID was downloaded as the structure of tyrosinase. The downloaded enzyme was then energy-reduced through the amber force field (gradient algorithm) using software named UCSF chimera 1.10.1 [27]. The physical, chemical and computational application, Ramachandran chart of the enzyme was examined by the Molprobiy database and Ramachandran chart was drawn using Discovery studio 2.1 software. The enzyme physical structure and mathematical calculation were determined using VADAR 1.8 [28].

Grid generation and molecular docking

The molecular docking experiment was initiated, a protein preparation wizard was used for the optimization of the enzyme structure. Mandatory atoms were added into the protein structure. The enzyme then reduced to obtain the joint root mean square deviation (RMSD) of 0.3 Å with an optimum force field. The published literature was used for the identification of the dynamic region of protein from the crystal structure of the obtained protein [29,30]. Glide docking method was used for docking experiments of ACE with mushroom tyrosinase [23]. ACE was projected using the ligprep method in the

Schrödinger Suite. The anticipated binding dynamisms (docking scores) and conformational locations of ACE within the energetic area of tyrosinase were also executed using Glide testing. During the docking or simulations, both fractional plasticity and full plasticity around the energetic location deposits are implemented by Glide/SP/XP and induced fit docking (IFD) methods [22].

Molecular dynamics (MD) simulations assay

A Molecular dynamics simulation was performed using GROMCS 4.5.4 package for verification of docking clusters [31]. The ACE topology was investigated using the online PRODRG database and enzyme topology was examined using GROMOS 53A6 [32]. Additionally, the receptor-ligand multiplexes were solvated and positioned in the cubic packet middle having adjusted 9 Å distance. The system charge was neutralized by adding ions. Energy reduction (nsteps=50,000) was ended by the sharpest lineage technique (1000 ps). While energy measurements were prepared by Particle Mesh Ewald (PME) technique [33], The covalent bond restraints were considered by the linear constraint solver (LINCS) algorithm [34]. Finally, MD arrangement is saved in md.mdp file, period phase for integration was accustomed as 0.002 ps, while the final distance for small range neighbor list (rlist) was attuned at 0.8 nm. The MD run was set to 30,000 ps with nsteps 15,000,000 for protein- drug complex and trajectories files analysis was done by Xmgrace tool using diverse instructions such as 'g_energy', 'g_rms', 'g_rmsf', 'g_chi', 'g_sas', and 'g_gyrate' respectively, (<http://plasma-gate.weizmann.ac.il/Grace/>).

Results and Discussion

Enzyme inhibition

The enzyme inhibition of ACE was performed against tyrosinase. To determine the IC₅₀ values of ACE and kojic acid, eight concentrations were used. The ACE showed a very good IC₅₀ assessment (0.353 $\pm$ 0.003 μ M) as equated to that of positive control kojic acid (16.841 $\pm$ 1.161). (Fig. 1A-D). Thus, it was envisaged that the effect may be due to the multifunctional nature of ACE structure that makes this molecule prone to establish some superb connections with the energetic side of the target protein.

Figure 1. IC_{50} calculations of ACE and positive control

Kinetic analysis

Kinetic experiments were carried out to reveal the inhibition pattern of ACE on tyrosinase. The mechanistic outcomes of tyrosinase can be identified the well-known plot Lineweaver-Burk

of $1/V$ against $1/[S]$. Various straight lines were calculated in the presence of ACE doses. The findings of above mentioned plot of ACE declared that V_{max} stays constant without notable alterations on the slope. These findings confirmed that ACE blocked the studied enzyme competitively (Fig. 2A). In addition, the enzyme inhibition dissociation constant, K_i was determined using ACE doses against the slope (Fig. 2B) and is calculated as 0.08 μM .

Figure 2. The enzyme mechanistic analysis of ACE (A) ACE versus substrate (B) slope against ACE doses

In vivo zebrafish depigmentation assay

Zebrafish is vital to research model against various diseases, due to gene makeup similarity with human [20]. Owing to these benefits, the zebrafish embryos were used to determine the depigmentation efficacy of ACE through *in vivo* assay. The inhibition efficacy of ACE on the coloring of zebrafish was investigated with the treatment of 5, 10, 15 and 20 μM of inhibitor ACE and equal doses were tested for kojic acid. The pigment level of zebrafish significantly decreased $P < 0.001$ (Fig. 3) to about

62.45% while positive control kojic acid showed 38.33% at 20 μM . Additionally, inhibitor ACE exhibited good depigmentation effects at 10 and 20 μM treatment, relative to those of kojic acid.

Figure 3. Depigmentation efficacy of ACE in zebrafish (A) showed the rapid decrease in fish coloring from black brown to whitish at various doses (B) the fish image color intensity were measured using ImageJ software and compared using t-test. *P < 0.05; ** P < 0.001.

Melanin quantification

Fingerling zebrafishes were treated with various doses of ACE and after their fixed experimental timings the animals were homogenized and the crude extract was prepared for melanin content calculations. Findings declared that contents of melanin were notable reduced ($P < 0.001$) in the

ACE experimental animals at 20 μM in comparison to non-experimental animals and standard kojic acid in experiments. Melanin contents were reduced to minimum quantity in case of zebrafish embryos treated with kojic acid, while these were decreased to a relatively greater extent in the case of ACE treated embryos (Fig. 4).

Figure 4. Melanin quantification after treatment with ACE and kojic acid along with non-treated embryos. *P < 0.05; ** P < 0.001.

Molecular docking analysis

Binding energy calculation of ACE

The computation method i.e. docking the experimental compound with the inhibited enzyme is a very important strategy to investigate the binding capacity and pattern [21, 22]. To calculate the binding energy value of ACE within the energetic site of enzyme (Tyrosinase), the computational docked mixtures of tyrosinase and ACE were investigated through docking energy score and bonding connections and versions. The interaction energy scores exhibited the optimum confirmation side within the dynamic side of tyrosinase. The docking findings declared that ACE possesses an excellent glide score (-7.306Kcal/mol).

Ligand-Binding analysis of tyrosinase docked complexes

Bioinformatics (docking) revealed that ACE involves in the active interaction site of tyrosinase with various favorable postures. The binding conformational analysis exhibited 2 energetic hydrogen bonds present in the ACE-tyrosinase docking complex at different residual

positions, i.e. His244 and Glu322. The oxygen and hydrogen atoms of ACE formed hydrogen bonds with His244 or Glu322 with bond distance of 1.64 and 1.47 Å, respectively. The bond distances in the ACE-tyrosinase docking complex were appropriate as it is comparable with the standard value (3.0 Å). Moreover, a couple of π - π connections were detected between ACE and His244 and Phe264 deposits of a target protein. Prior docking studies showed that the significance of these amino acids in bonding with other tyrosinase inhibitors supports our docking results [23]. The 3D and 2D pictorial presentation of ACE-tyrosinase docking complexes is shown in (Fig. 5A-B).

Figure 5. Docking complex of ACE (A)3D and (B)2D within the energetic section of objective protein

Molecular Dynamic Simulations

Root mean square deviation and fluctuation (RMSD/RMSF) analysis

Examination of residual elasticity of receptor through MD simulation RMSD and RMSF diagrams were done to determine the tyrosinase framework backbone conduct. The RMSD graph result of ACE interprets the protein residual deviation in a 30 ns simulation period. Initially, the graph line showed an increasing trend from 0-2500 ps having RMSD rate series from 0.15-0.25 nm. From 2500 to 10,000 ps a minute firm and graph line was showed with constant RMSD 0.2 nm. The chart line showed a slight increment between 10,000-15,000 ps but the RMSD value did not fluctuate much during this time frame. Finally, the chart exhibited a firm line during the remaining stimulation period of 15,000-30,000 ps (Fig. 6A). Furthermore, the generated RMSF chart of ACE docked complex (Fig. 6B), showed loop fluctuations within the target protein throughout the stimulation period.

Radius of gyration and SASA graphs of ACE docking complex at 30ns

Radius of gyration (Rg) and protein stability

Radius of gyration (Rg) analysis was performed against the ACE docked complex in order to analyze the solidity of the tyrosinase. The presented chart revealed the Rg rate sustained around 2 nm during the experimental period 0-30,000 ps. The Rg time diagram revealed that the enduring pillar and compact of receptor protein was gradually unchanged after attachment with ACE (Fig. 6C). The solvent-accessible area (SASA) analysis revealed that no conformational fluctuations were observed in the simulation phase. The SASA assessment remained stable in the whole simulation at 125 nm which depicted that the docking complex remains stable and no more fluctuations were seen in protein conformations (Fig. 6D).

Figure 6. RMSD (A and B), and RMSF (C and D) graphs of ACE docking complex at 30ns.

Conclusion

In summary, ACE exhibited excellent activity having the IC_{50} value ($0.353 \pm 0.003 \mu M$) in comparison with the standard kojic acid ($16.841 \pm 1.161 \mu M$). In *in vivo* study ACE showed good results at the concentration of $20 \mu M$ as compared to standard and significantly reduced the level of pigmentation. *In silico* studies of ACE also justified the strong interaction with tyrosinase and showed good stability. Therefore, ACE, in particular, might serve as an excellent agent for the treatment of skin related diseases.

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Disclosure Statement

The authors have no conflict of interest.

References

1. T. Pillaiyar, M. Manickam and S. H. Jung, *Cell. Signal.*, 40 (2017) 99.
<https://doi.org/10.1016/j.cellsig.2017.09.004>
2. M. Brenner and V. J. Hearing, *Photochem. Photobiol.*, 84 (2008) 539.
<https://doi.org/10.1111/j.1751-1097.2007.00226.x>
3. J. Bonaventure, M. J. Domingues and L. Larue, *Pigm. Cell Melanoma Res.*, 26 (2013) 316.
<https://doi.org/10.1111/pcmr.12080>
4. J. Borovanský and I. Wiley, John Wiley Distributor c2011, Weinheim, Baden-Wurttemberg, Germany, (2011). ISBN: 978-3-527-32892-5.
<https://doi.org/10.1002/9783527636150.ch12>
5. T. C. Lei, V. Virador, K. I. Yasumoto, W. D. Vieira, K. Toyofuku and V. J. Hearing, *J. Invest. Dermatol.*, 119 (2002) 1341.
<https://doi.org/10.1046/j.1523-1747.2002.19607.x>

6. E. V. Sviderskaya, S. P. Hill, D. Balachandar, G. S. Barsh and D. C. Bennett, *Dev. Dynam.*, 221 (2001) 373.
<https://doi.org/10.1002/dvdy.1153>
7. G. E. Costin and V. J. Hearing, *FASEB J.*, 21 (2007) 976.
<https://doi.org/10.1096/fj.06-6649rev>
8. J. Y. Yang, J. H. Koo, Y. G. Song, K. B. Kwon, J. H. Lee, H. S. Sohn, B. H. Park, E. C. Jhee, J. W. Park, *Acta Pharmacol. Sin.*, 27 (2006) 1467.
<https://doi.org/10.1111/j.1745-7254.2006.00435.x>
9. S. K. Kim, S. J. Oh, S. Y. Park, W. J. Kim, Y. S. Kim and Y. C. Kim, *Pigment. Cell Melanoma Res.*, 31 (2018) 277.
<https://doi.org/10.1111/pcmr.12658>
10. S. M. Hashemi and E. Saeed, *Pharm. Biomed. Res.*, 1 (2015) 1.
<https://doi.org/10.18869/acadpub.pbr.1.1.1>
11. C. Serre, V. Busuttill and J. M. Botto, *Int. J. Cosmet. Sci.*, 40 (2018) 328.
<https://doi.org/10.1111/ics.12466>
12. S. Ito, *Pigment Cell Res.*, 16 (2003) 230.
<https://doi.org/10.1034/j.1600-0749.2003.00037.x>
13. A. Agalou, M. Thrapsianiotis, A. Angelis, A. Papakyriakou, A.L. Skaltsounis, N. Aligiannis and D. Beis, *Front. Pharmacol.*, 9 (2018) 265.
<https://doi.org/10.3389/fphar.2018.00265>
14. J. Rennekamp and R. T. Peterson, *Curr. Opin. Chem. Biol.*, 24 (2015) 58.
<https://doi.org/10.1016/j.cbpa.2014.10.025>
15. N. Tabassum, H. Tai, D. W. Jung and D. R. Williams, *Evid. Based. Complement. Alternat. Med.*, 2015 (2015) 1.
<https://doi.org/10.1155/2015/287847>
16. A. MacRae and R. T. Peterson, *Nat. Rev. Drug Discov.*, 14 (2015) 721.
<https://doi.org/10.1038/nrd4627>
17. R. Leenaraj, D. Manimaran and I. H. Joe, *J. Mol. Struct.*, 1123 (2016) 180.
<https://doi.org/10.1016/j.molstruc.2016.06.035>
18. T. M. Shehata, M. H. Abdallah and M. M. Ibrahim, *AAPS. Pharm. Sci. Tech.*, 16 (2015) 375.
<https://doi.org/10.1208/s12249-014-0233-5>
19. R. Qamar, A. Saeed, F.A. Larik, Q. Abbas, M. Hassan, H. Raza and S.Y. Seo, *Chem. Biol. Drug Des.*, 93 (2019) 123.
<https://doi.org/10.1111/cbdd.13352>
20. Q. Abbas, Z. Ashraf, M. Hassan, H. Nadeem, M. Latif, S. Afzal and S.Y. Seo, *Drug Des. Devel. Ther.*, 11 (2017) 2029.
<https://doi.org/10.2147/dddt.s137550>
21. Q. Abbas, H. Raza, M. Hassan, A. R. Phull, S. J. Kim and S. Y. Seo, *Chem. Biodiversity*, 14 (2017) e1700117.
<https://doi.org/10.1002/cbdv.201700117>
22. F. A. Larik, A. Saeed, P. A. Channar, U. Muqadar, Q. Abbas, M. Hassan, S. Y. Seo and M. Bolte, *Eur. J. Med. Chem.*, 141 (2017) 273.
<https://doi.org/10.1016/j.ejmech.2017.09.059>
23. A. Saeed, P. A. Mahesar, P. A. Channar, Q. Abbas, F. A. Larik, M. Hassan, H. Raza and S. Y. Seo, *Bioorg. Chem.*, 74 (2017) 187.
<https://doi.org/10.1016/j.bioorg.2017.08.002>
24. T. Y. Choi, J. H. Kim, D. H. Ko, C. H. Kim, J. S. Hwang, S. Ahn, S. Y. Kim, C. D. Kim, J.H. Lee and T. J. Yoon, *Pigment Cell Res.*, 20 (2007) 120.
<https://doi.org/10.1111/j.1600-0749.2007.00365.x>
25. K. D. Hsu, H. J. Chen, C. S. Wang, C. C. Lum, S. P. Wu, S. P. Lin and K. C. Cheng, *Sci. Rep.*, 9 (2016) 32854.
<https://doi.org/10.1038/srep32854>
26. S. H. Baek and S. H. Lee, *Exp. Dermatol.*, 24 (2015) 761.
<https://doi.org/10.1111/exd.12765>
27. F. Pettersen, T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng and T. E. Ferrin, *J. Comput. Chem.*, 25 (2004) 1605.
<https://doi.org/10.1002/jcc.20084>
28. L. Willard, A. Ranjan, H. Zhang, H. Monzavi, R. F. Boyko, B. D. Sykes and D. S. Wishart, *Nucleic Acids Res.*, 31 (2003) 3316.
<https://doi.org/10.1093/nar/gkg565>
29. R. A. Friesner, R. B. Murphy, M. P. Repasky, L. L. Frye, J. R. Greenwood, T. A. Halgren, P. C. Sanschagrin and D. T. Mainz, *J. Med. Chem.*, 49 (2006) 6177.
<https://doi.org/10.1021/jm051256o>

30. R. Farid, T. Day, R. A. Friesner and R. A. Pearlstein, *Bioorg. Med. Chem.*, 14 (2006) 3160.
<https://doi.org/10.1016/j.bmc.2005.12.032>
31. S. Pronk, S. Páll, R. Schulz, P. Larsson, P. Bjelkmar, R. Apostolov, M. R. Shirts, J. C. Smith, P. M. Kasson, D. Van Der Spoel and B. Hess, *Bioinformatics*, 29 (2013) 845.
<https://doi.org/10.1093/bioinformatics/btt055>
32. A.W. Schüttelkopf and D. M. F. Van Aalten, *Acta Crystallogr. D Biol. Crystallogr.*, 60 (2004) 1355.
<https://doi.org/10.1107/s0907444904011679>
33. H. Wang, F. Dommert and C. Holm, *J. Chem. Phys.*, 133 (2010) 034117.
<https://doi.org/10.1063/1.3446812>
34. S. Amiri, M. S. Sansom and P. C. Biggin, *Protein Eng. Des. Sel.*, 20 (2007) 353.
<https://doi.org/10.1093/protein/gzm029>