April 1, 2020

Editor in Chief
Indonesian Journal of Chemistry

First of all, we would like to thanks to reviewers for their constructive comment, and time spent to analyze this manuscript. The responses and explanations related to their comments for our manuscript are listed below.

Reviewer 2

1. "From statistical analysis, we obtain r^2 of training and test sets were 0.91 and 0.70, respectively, while q^2 of leave-one-out cross-validation was 0.56" in Abstract <= Deviation of r^2 and q^2 of more than 0.2 IS an indications of outliers and overfitting (Please see https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1241620/pdf/ehp0111-001361.pdf). Since the authors argued that "According to the results, we do not think that the high deviation indicates overfitting because both of r2 and q2, as internal validation, were calculated by using training data.", please provide the y-scrambling method to validate that there is no outlier or overfitting.

   Answer:
   Thank you for your correction. We are also curious why the difference of r^2 and q^2 is so large. So, we re-calculate all of the validation parameters and found a mistake in the calculation. we have revised the value of the validation parameter by using the correct one. Also, to verify that the model is not overfitting, we add another parameter ${}^{c}R_p^2$ that represents the comparison of the correlation coefficient between randomized and non-randomized model. We found that the value of ${}^{c}R_p^2$ parameter of our model (0.76) is more than threshold (0.5), which indicates that our model is not overfitting.

$$R_p^2 = R \times \sqrt{R^2 - R_r^2} \qquad (13)$$

where y and $\hat{y}$ represent the experimental and predicted value of pIC_{50}, while $\bar{y}$ and $\bar{\hat{y}}$ represent tl average of the experimental and predicted value. The value of R_p^2 represents the correlati coefficient that is calculated by considering randomized and non-randomized model. This paramet can be used to verify that the model is not overfitting. The acceptability of the model was consider according to the following criteria[23]–[25]

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$$R_p^2 > 0.5$$

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as provided in Table 2, met the criteria. These results indicate that the CoMFA model was valid an acceptable. Also, we found that the value of R_p^2 is larger than 0.5, which indicates that the mod is not overfitting.

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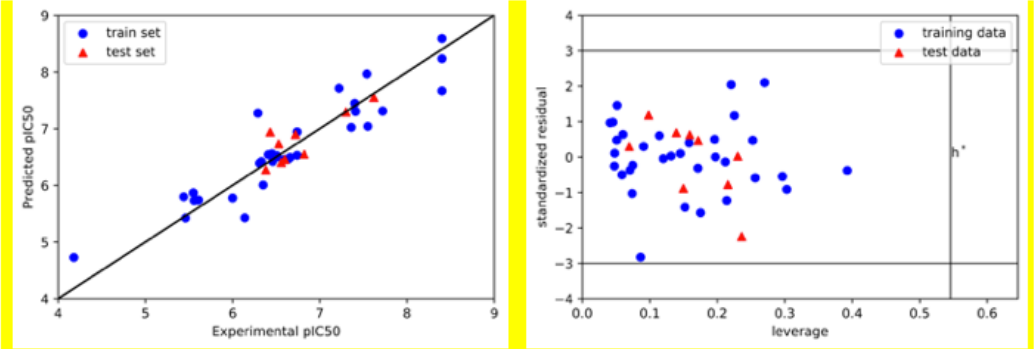

(a) (b)

Table 2. Calculated statistical parameter of CoMFA model.

Parameter	Training set	Test set	Threshold[23]–[25]
R^2	0.85	0.70	> 0.6
Q^2	0.77	-	> 0.5
k'	0.86	1.05	$0.85 \leq k' \leq 1.15$
$\frac{(r^2 - r_0^2)}{r^2}$	0.07	0.00	< 0.1
$\lvert r_0^2 - r_0'^2 \rvert$	0.05	0.09	< 0.3
$\overline{r_m^2}$	0.72	0.57	> 0.5
Δr_m^2	0.14	0.18	< 0.2
$^{c}R_p^2$	0.76	-	> 0.5

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2. The author, without any references, argued that "According to alignment analysis, we found that RMSD value is 2.11 Å, that is low enough to confirm the validity of the docking procedure." It is already established that the RMSD should be as low as possible and should not exceed 2.0 angstrom. Please see https://www.ncbi.nlm.nih.gov/pubmed/17238265. The docking method presented in this manuscript is, therefore, NOT VALID. Hence, all docking results are NOT VALID. The authors should modify the docking parameters to reach the acceptable validity.:

Answer:
Thank you for your fundamental correction. To obtain a valid docking result, we have make several change regarding the docking protocol. Firstly, we use smina docking program instead of autodock vina, as we found the smina program produce the ligand structure that more resemble the native structure. Secondly, we re-define the box size by using the native ligand position as the center and expanded the box size of 8 Angstrom. By using the new protocol, we found the RMSD value between docked ligand and the native one become 1.09 Angstrom, that is lower than the threshold (2.0). Then, we restart the docking simulation by using the validated protocol.

protein data bank[28]. The binding site of the receptor was identified from the position of native cycloguanil found in the X-ray structure. However, we removed the original cycloguanil from the structure as part of the preparation process.

To construct pdbqt file of the receptor, we use Open Babel package[17] to add polar hydrogens and assign Gagstier charge to the ligand. The grid box, that define the docking area, was constructed by using the native ligand position as the center and expanded the box with the size of 8 Å. The docking simulation was performed by using Smina docking package[29] and the binding pose obtained from the docking simulation was plotted by using LigPlot package[30].

The docking procedure was validated by extracting native cycloguanil ligand from wild-type *Plasmodium falciparum* DHFR-TS complex and re-docking the ligand to the receptor. The validity of the method was determined by aligning the ligand obtained from docking simulation and original X-ray crystal structure, and calculating the deviation between both structures. In this case, the

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corresponds to the increase of the activity. The greater activity of c26 compare to that of c22 is related to the existence of Cl^- substituent in c26 that is more negative than hydrogen of c22.

Molecular Docking

Molecular docking analysis was carried out on the most active compound (c33) and the least active compound (c8). We found that the calculated binding score for c33 and c8 compound are -10.7 kcal/mol and -7.9 kcal/mol, respectively. The lower value of the binding score indicated that

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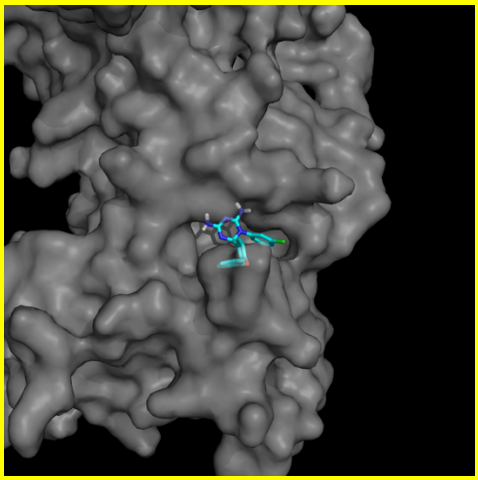
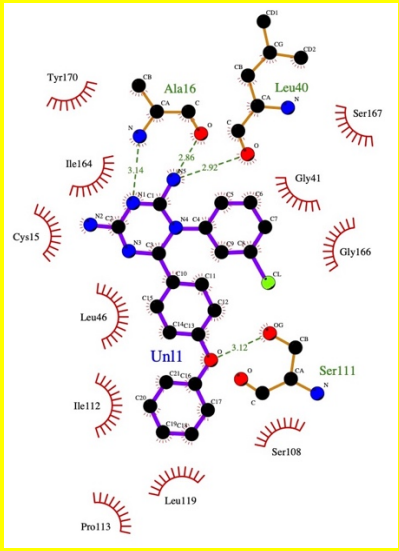


(a)



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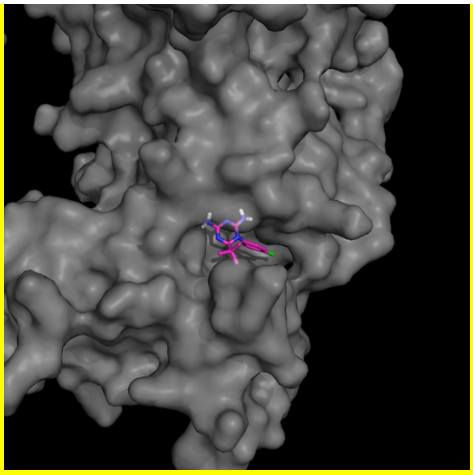
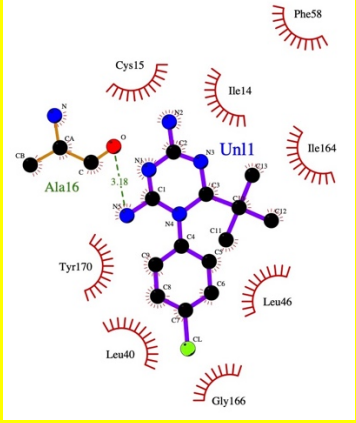


(c)



(d)

Figure 5. The binding poses of (a) c33 and (c) c8 that are docked into 3UM8 binding site, and the plot of ligand-receptor interaction of (b) c33 and (d) c8 ligands.

The 2D plots of ligand-receptor interaction between c33 and c8 compounds with 3UM8 receptor were presented in **Error! Reference source not found.** and 5(d). The number of hydrogen bonds found in the interaction of the receptor with c33 and c8 are four and one, respectively. As for c33 ligands, the hydrogen bonds were found in the interaction of the ligands with ALA16, LEU40 and SER111. As for c8 ligands, a hydrogen bond was found in the interaction of the ligands with ALA16. From the docking results, we found that the higher number of hydrogen bonds found in c33-receptor interaction contribute to the high activity of the ligand.

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between the ligand configuration obtained from docking simulation and the original X-ray crystal structure, in which the alignment is shown in Figure 6. According to validation analysis, we found that the RMSD value is 1.09 Å, which is low enough to confirm that the docking procedure is valid[34].

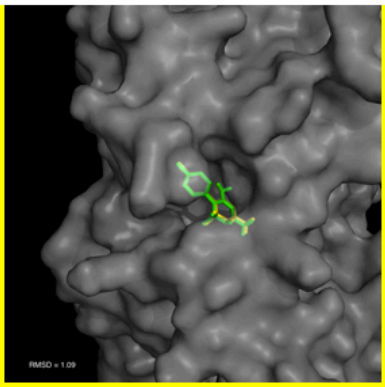

Figure 6. The alignment of the ligand configuration obtained from docking simulation (green) and original X-ray crystal structure (yellow)

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3. Since the docking results are NOT VALID, the MD simulations are also NOT VALID. Please redo the MD simulations after using the valid docking method to put the tested compounds inside the binding pocket.

Answer:
By using the docked structure obtain from the docking simulation, we restart the MD simulation. However, we found that the result is just slightly different from the previous one. In the simulation, we change the force field (FF) become CHARMM36, as this force field is one of FF that is recommended for derive the topology of ligand. Also, we extended the NVT and NPT simulation become 1 ns for both simulations.

molecular dynamics simulation for the complex system. The molecular dynamics simulation of ligand-protein complexes was performed by using Gromacs 2018 package[31].

The preparation of structure of the complex was carried out by using MacPymol package[27]. The topology of protein was prepared by using CHARMM36 force field. Meanwhile, the topology of ligand was estimated by using CHARMM General Force Field (CGenFF) server (https://cgenff.umaryland.edu/). Then, the complex was solvated into dodecahedron box of SPC water with a distance of 1.00 nm from the molecule to the edge of box. The solvated complex system was neutralized by replacing the solvent molecule with Cl^- ions.

After completing the preparation step, the system was minimized by using steepest descent algorithm, followed by consecutive NVT (1 ns) and NPT (1 ns) equilibration. During the equilibrations, the temperature was fixed at 300 K by utilizing V-rescale thermostat algorithm[32], and the pressure was fixed at 1.0 bar by utilizing Berendsen barostat algorithm[33]. Finally, MD

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Molecular Dynamics

To confirm the stability of the solvated complex system, we analyzed the fluctuation of RMSD and the radius of gyration for 20 ns simulation of both complex, as shown in Figure 7(a). RMSD analysis shows that both of c33 and c8 complex reaches the equilibrium state after 10 ns. Also, we found that RMSD value of c33 complex is slightly lower than that of c8 complex, as an indication of the better stability of c33 complex. The fluctuation of radius of gyration, that indicate the compactness of the complex system, was provided in Figure 7(b). We found that the compactness of the c33 complex is lower than that of the c8 complex.

We also analyzed the fluctuation of the hydrogen bond formed during the simulation, as shown in Figure 7(c). We found that the hydrogen bond formed during the simulation of the c33 complex is more than that of the c8 complex. In the case of c33 complex, the maximum number and the average number of hydrogen bond are 4 and 0.78, respectively. Meanwhile, in the case of the c8 complex, the maximum number and the average number of hydrogen bond are 3 and 0.82, respectively. According to the results, it seems that the number of hydrogen bond formed during the simulation is quite similar between both complexes.

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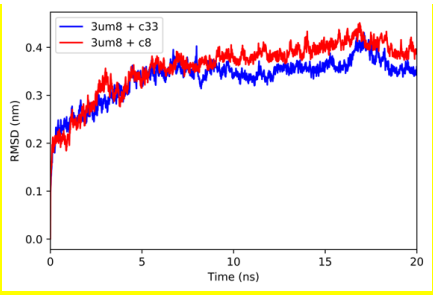
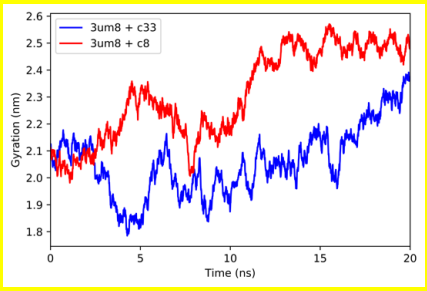


(a)



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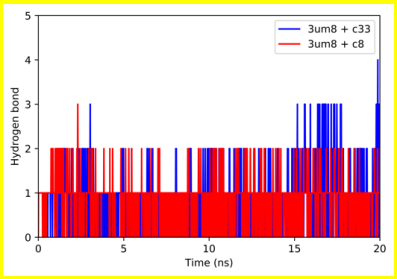

(c)

Figure 7. Plots of (a) RMSD, (b) radius of gyration and (c) hydrogen bonds number from MD simulation of c33 and c8 complex.

Finally, we align the final structure of the complex obtained from MD simulation with the initial structure to verify that the structure did not change significantly during the simulation. The results of alignment for both complexes were presented in Figure . We found that the final structure for both complexes resembles the initial structure with a small deviation. This is indicated by the low RMSD that is evaluated from the alignment processes, which the value 1.657 Å and 1.354 Å for the complex with c33 and c8, respectively. This points out that the structure did not change significantly during the simulation.

(a)

(b)

Figure 8. The alignment of the initial (yellow) and final (red) structure obtained from MD simulation of 3UM8 complex with (a) c33 and (b) c8 ligand

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We really appreciate for the referee's comments. We believed that our manuscript had been improved after considering the constructive criticisms from the referee.

Your sincerely,
Isman Kurniawan on behalf of all authors.