Indonesian J. Pharm. Vol. 27 No. 3 : 163 – 173
ISSN-p : 2338-9427
DOI: 10.14499/indonesianjpharm27iss3pp163

Research Article

OPTIMIZING STRUCTURE-BASED VIRTUAL SCREENING PROTOCOL TO IDENTIFY PHYTOCHEMICALS AS CYCLOOXYGENASE-2 INHIBITORS

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Submitted: 14-04-2016
Revised: 13-06-2016
Accepted: 28-07-2016

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ABSTRACT

By employing Databases of Useful Decoys (DUD) and its enhanced version (DUD-E), several attempts to construct validated Structure-based Virtual Screening (SBVS) protocols to identify cyclooxygenase-2 (COX-2) inhibitors have been performed. Both databases tagged active COX-2 inhibitors for compounds with IC_{50} values < 1μM. In the search for phytochemicals as natural COX-2 inhibitors, however, most of their IC_{50} values are in the micromolar range, which will likely be identified as non-inhibitors for COX-2 by the available SBVS protocols. In this article, validation of an SBVS protocol by adding marginal active COX-2 inhibitors from DUD-E as active compounds is presented. Binary quantitative-structure activity relationship analysis by using recursive partition and regression tree method was performed subsequently to optimize the predictive ability of the protocol. The enrichment factor and the *F*-measure values of the optimized protocol could reach 44.78 and 0.47, respectively. The optimized protocol could identify 1 out of 9 phytochemicals as COX-2 inhibitors.

Key words: Structure-based virtual screening (SBVS), phytochemical, cyclooxygenase-2 (COX-2).

INTRODUCTION

Enzyme cyclooxygenase-2 (COX-2) plays important role in several inflammation-related pathophysiological processes (Chakraborti *et al.*, 2010; Penning *et al.*, 1997; Willoughby *et al.*, 2000). Moreover, besides being employed in the therapy for inflammation, a blockbuster COX-2 selective inhibitor celecoxib (Maggon, 2005; Penning *et al.*, 1997; Sadée and Bohn, 2006) was reported could interfere the apoptosis pathways in cancer (Jendrossek, 2013), bind to estrogen receptor alpha (Dai *et al.*, 2012; Istyastono *et al.*, 2015[a]) and reducing scar formation during wound healing processes (Wilgus *et al.*, 2004, 2003). Targeting COX-2 in drug discovery and development programs has therefore become of considerable interest, which has also been shown by several attempts to construct validated *in silico* protocols, including Structure-Based Virtual Screening (SBVS) protocols, and to employ the protocols to identify and design potent inhibitors for COX-2, not only by academia but also in companies related to drug discovery and development (Cappel *et al.*, 2015; Chakraborti *et al.*, 2010; Cianchi *et al.*, 2005; Kaserer *et al.*, 2015; Krüger and Evers, 2010; Larsson *et al.*, 2005; Pany *et al.*, 2013; Rao *et al.*, 2006; Yuniarti *et al.*, 2012). Together with the public availability of the COX-2 crystal structure by Kurumbail *et al.* (1996) followed by other novel crystal structures of COX-2 with different co-crystal ligands (Rowlinson *et al.*, 2003; Wang *et al.*, 2010[a]; Wang *et al.*, 2010[b]), the publicly available Databases of Useful Decoys (DUD) (Huang *et al.*, 2006) and the enhanced version of DUD (DUD-E) (Mysinger *et al.*, 2012) could serve as the sources of virtual targets, ligands and decoys to construct and retrospectively validate SBVS protocols to identify COX-2 inhibitors (Huang *et al.*, 2006; Mysinger *et al.*, 2012; Yuniarti *et al.*, 2011). Notably, both DUD and DUD-E required a compound could be identified as a potent COX-2 inhibitor if the compound showed IC_{50} as a COX-2 inhibitor < 1μM (Huang *et al.*, 2006; Mysinger *et al.*, 2012).

The search for COX-2 inhibitors has also involved natural products (Orlikova *et al.*, 2013; Pany *et al.*, 2013). Nevertheless, two most

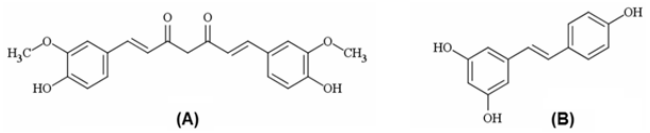

Figure 1. Structures of curcumin, the active substance found in turmeric (*Curcuma longa*) **(A)** and resveratrol, a compound mainly found in grapes and red wine **(B)** (Orlikova *et al.*, 2013; Setyaningsih *et al.*, 2013; Yuniarti *et al.*, 2012)

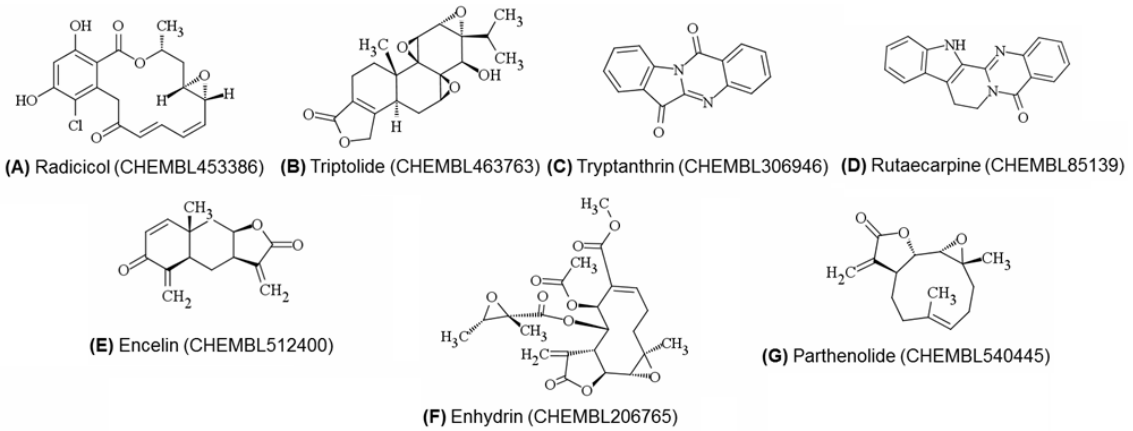

Figure 2. Structures of phytochemicals published in Journal of Natural Products and stored in ChEMBL_21 database that have IC_{50} values as COX-2 inhibitors < 1 μM and do not violate the Lipinski's rule of 5 (Bento *et al.*, 2014).

well-known natural COX-2 inhibitors curcumin (Figure 1A) and resveratrol (Figure 1B) showed IC_{50} values of 79.2μM and 32.0μM, respectively (Gautam *et al.*, 2011; Larsson *et al.*, 2005). By using the method employed by Istyastono *et al.* (2015[b]) to have additional active histamine H_4 receptor, the data of compounds that have been tested as COX-2 inhibitors which have been stored in ChEMBL version 21 (ChEMBL_21; https://www.ebi.ac.uk/chembl/) (Bento *et al.*, 2014) were downloaded and examined. By taking into account only compounds that published in Journal of Natural Products (http://pubs.acs.org/journal/jnprdf), it was recorded that at least 74 phytochemicals have been examined as COX-2 inhibitors (Bento *et al.*, 2014). Similar to curcumin and resveratrol, most of those 74 phytochemicals are however marginal COX-2 inhibitors with IC_{50} values > 1μM, which will unlikely identified as COX-2 inhibitors by SBVS protocols validated using data from DUD or DUD-E (Bento *et al.*, 2014; Huang *et al.*, 2006; Mysinger *et al.*, 2012). In fact, only 9 compounds out of the 74 phytochemicals that have IC_{50} values as COX-2 inhibitors < 1μM (Bento *et al.*, 2014). Moreover, only 7 out of those 9 compounds that meet the Lipinski's rule of 5 (Figure 2) (Lipinski *et al.*, 2001). Therefore, development of validated SBVS protocols that can identify marginal and potent COX-2 inhibitors to cover phytochemicals as potential lead compounds is required.

The research presented in this paper was aimed to construct and retrospectively validate SBVS protocols to identify marginal to potent COX-2 inhibitors by using data from DUD-E with additional marginal active COX-2 inhibitors from DUD-E as active compounds (Mysinger *et al.*, 2012). The protocols were constructed by employing PLANTS1.2 as the molecular docking software (Korb *et al.*, 2007; Korb *et al.*, 2009) and PyPLIF to identify Protein-Ligand Interaction Fingerprints (PLIF) to COX-2 as the re-scoring functions (Radifar *et al.*, 2013[a]; Radifar *et al.*, 2013[b]). The quality of the SBVS protocol was subsequently assessed (Cannon *et al.*, 2007; de Graaf *et al.*, 2011; Desaphy *et al.*, 2013; Powers, 2011) and

compared to the original SBVS accompanying the release of DUD-E (Mysinger *et al.*, 2012). Very recently, Istyastono (2015) showed that using recursive partition and regression tree (RPART) method with the PLANTS1.2 docking score (ChemPLP score) and the PLIF bitstrings resulted from PyPLIF as the descriptors increased significantly the SBVS predictive ability (Istyastono, 2015; Therneau *et al.*, 2015). This approach could avoid the dependency of the application of PyPLIF towards the reference compound (Istyastono, 2015). By employing the same strategy, this research could increase significantly the predictive ability of the SBVS protocols to identify marginal and potent COX-2 inhibitors with enrichment factor (EF) value of 44.78. The optimized protocol was subsequently employed to virtually screen phytochemicals in figures 1 and 2 as COX-2 inhibitors.

MATERIALS AND METHODS

The crystal structure of COX-2 obtained from the protein data bank (PDB) with PDB id of 3LN1 (Wang *et al.*, 2010[a]) was used as the reference structure. Active (435 compounds) and marginal active (1538 compounds) COX-2 inhibitors and the decoys (23150 compounds) from DUD-E (Mysinger *et al.*, 2012) were employed to perform retrospective validations for the SBVS protocol. All calculations and computational simulations were performed on a Linux (Ubuntu 12.04 LTS Precise Pangolin) machine with Intel(R) Xeon(R) CPU E31220 (@ 3.10 GHz) as the processors and 8.00 GB of RAM. Computational medicinal chemistry applications employed in this research were SPORES (ten Brink and Exner, 2009), PLANTS1.2 (Korb *et al.*, 2007; Korb *et al.*, 2009), Open Babel 2.2.3 (O'Boyle *et al.*, 2011), PyPLIF 0.1.1 (Radifar *et al.*, 2013[a]; Radifar *et al.*, 2013[b]), and PyMOL 1.2r1 (Lill and Danielson, 2011). Statistical analysis was performed by using R 3.2.3 (R Core Team, 2015).

Computational Methods

Virtual molecular target preparation

The crystal structure of COX-2 with the PDB id of 3LN1 (Wang *et al.*, 2010[a]) was downloaded from http://www.rcsb.org/pdb/explore.do?structureId=3ln1. Only chain A of the crystal structure used further in this research (Mysinger *et al.*, 2012). The module *splitpdb* in SPORES was subsequently used to split and to convert the splitted files into *mol2* files the virtual COX-2 (*protein.mol2*), the co-crystal ligand celecoxib (*ligand_CEL682_0.mol2*), and the water molecules. The *mol2* files were then ready to be employed in molecular docking simulation employing PLANTS1.2 docking software.

Ligands preparation for retrospective virtual screening

Known COX-2 active and marginal inhibitors and the decoys were downloaded in their SMILES format from DUD-e (Mysinger *et al.*, 2012). They were stored locally as *actives_final.ism*, *marginal_actives_nM_chembl.ism* and *decoys_final.ism*. Each compound in the files was then subjected to Open Babel 2.2.3 conversion software to be converted in its three dimensional (3D) format at pH 7.4 as a *mol2* file. The *settypes* module in SPORES was subsequently employed to properly check and assign the *mol2* file into a proper *mol2* file ready to dock by using PLANTS1.2 docking software.

Automated molecular docking and virtual screening

Similar to previously published procedures (Istyastono and Setyaningsih, 2015; Istyastono *et al.*, 2015[a]; Setiawati *et al.*, 2014), all virtual screenings were performed by docking program PLANTS1.2. For each compound, 50 poses were calculated and scored by the ChemPLP scoring function at speed setting 2. The binding pocket of COX-2 was defined by the coordinates of the center of the co-crystal ligand celecoxib and a radius of 5 Å (which is the maximum distance from the center defined by a 5 Å radius around the reference ligand). All other options of PLANTS1.2 were left at their default setting (Anita *et al.*, 2012; Istyastono and Setyaningsih, 2015). Every compound was virtually screened five times independently.

Rescoring using protein-ligand interaction fingerprints calculated by PyPLIF

Seven different interaction types (negatively charged, positively charged, hydrogen bond (H-bond) acceptor, H-bond donor, aromatic face-to-edge, aromatic face-to-

face, and hydrophobic interactions) were used to define the PLIF for each docking pose (Radifar *et al.*, 2013[b]; Setiawati *et al.*, 2014). The cavity used for the PLIF analysis consisted of a set of amino acid residues in the binding pocket of COX-2 defined in subsection Automated molecular docking and virtual screening.

Optimizing the SBVS predictive ability using RPART

The docking pose with the best ChemPLP score was selected for each virtually screened compound. The results were then ranked based on their ChemPLP score and the enrichment factor of True Positives (TP) at 1% False Positives (FP) value or the $EF_{1\%}$ value was calculated ($EF_{1\%}$ = %TP/$FP_{1\%}$) (de Graaf and Rognan, 2008; Istyastono and Setyaningsih, 2015). The compound was predicted as a COX-2 inhibitor if it showed ChemPLP score ≤ the ChemPLP score of the compound at 1% FP (Istyastono *et al.*, 2015[b]). Therefore, the protocol was encoded as the $EF_{1\%}$-ChemPLP based SBVS. By employing RPART package (R Core Team, 2015; Therneau *et al.*, 2015), decision trees were generated using ChemPLP score resulted from PLANTS1.2 (Korb *et al.*, 2009) and all PLIF bitstrings resulted from PyPLIF (Radifar *et al.*, 2013[b]) as the descriptors (Istyastono, 2015). The predictive quality of the best decision tree resulted from RPART method was measured by examining the EF value (de Graaf *et al.*, 2011), the balance accuracy (Cannon *et al.*, 2007; Therneau *et al.*, 2015) and F-measure value (Desaphy *et al.*, 2013). The predictive quality of the best decision tree was also compared to the predictive quality of the $EF_{1\%}$-ChemPLP based SBVS by employing McNemar's test (Cannon *et al.*, 2007).

Virtual screening on some phytochemicals

By employing the best SBVS protocol resulted from the subsection Optimizing the SBVS predictive ability using RPART, virtual screening campaigns to predict whether the COX-2 inhibitors (Figures 1 and 2) were also identified as COX-2 inhibitors virtually were performed. The virtual compounds were downloaded in their SMILE formats from the ChEMBL_21 database (Bento *et al.*, 2014). Subsequently, the virtual compounds were prepared and examined using the best SBVS protocol.

RESULTS AND DISCUSSION

Aimed to construct and evaluate the validation of an SBVS protocol to identify phytochemicals as inhibitors for COX-2, this research employed marginal and potent COX-2 inhibitors organized and stored in DUD-E (Mysinger *et al.*, 2012) as the retrospective compounds for the validation. Similar to the construction of an SBVS protocol to identify potent ligands for adrenergic β_2 receptor (Istyastono and Setyaningsih, 2015), the SBVS constructed here employed PLANTS1.2 as the molecular docking software (Korb *et al.*, 2007; Korb *et al.*, 2009) and PyPLIF as the PLIF identification software for rescoring the results from PLANTS1.2 (Radifar *et al.*, 2013[a]; Radifar *et al.*, 2013[b]). Additionally, the SBVS protocol constructed here employed the ChemPLP scores from PLANTS1.2 (Korb et al., 2009) and the PLIF bitstrings from PyLIF (Radifar *et al.*, 2013[b]) obtained from the docking pose with the best ChemPLP score in each virtually screened compound as descriptors to generate decision tress using RPART package in R (Istyastono, 2015; R Core Team, 2015; Therneau *et al.*, 2015).

Retrospective Validation of the SBVS protocol

The retrospective SBVS campaigns on COX-2 ligands and their decoys have resulted in 3,767,550 docking poses and 1,318,642,500 PLIF bitstrings resulted from 25,117 out of 25,123 screened compounds. Six decoys could not pass the constructed protocol, which were then assigned as True Negatives (TN). The $EF_{1\%}$-ChemPLP based SBVS resulted in ChemPLP score of -108.028 as the cutoff score. Employing this cutoff score resulted in a confusion matrix presented in Table 1. The EF value of the protocol was 3.55, which was very low compared to the reference (EF value = 12.9) and was not recommended to be employed further (Istyastono *et al.*, 2015[b]; Mysinger *et al.*, 2012). Inspired by Istyastono (2015), RPART package in R computational statistics software (R Core Team, 2015;

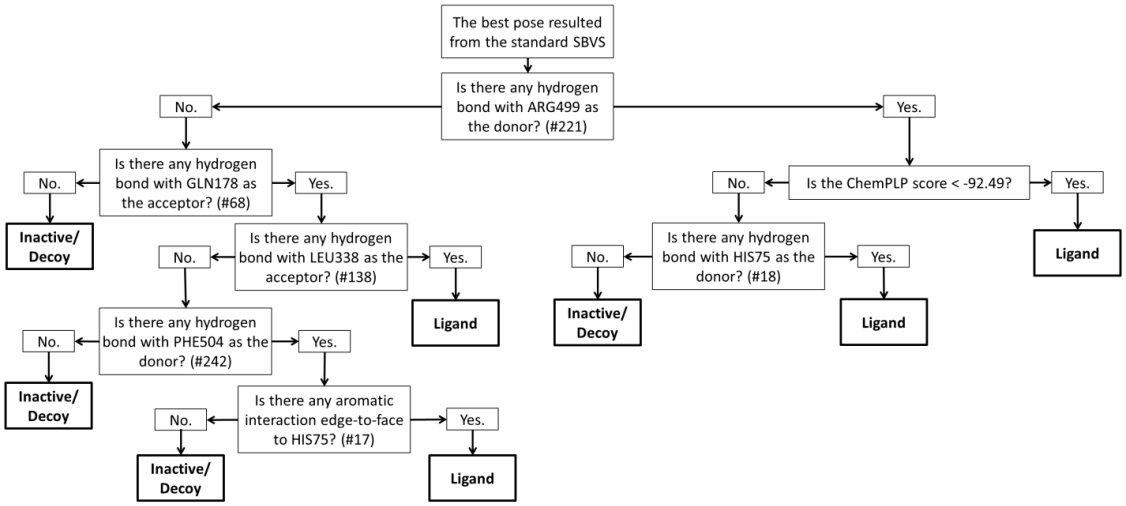

Figure 3. The decision tree adopted from the best decision tree resulted from the RPART method (Istyastono, 2015; Therneau *et al.*, 2015).

Table I. The confusion matrices and the statistical significances resulted from the validation of the SBVS protocol to identify COX-2 inhibitors

Parameters	SBVS	
	$EF_{1\%}$-ChemPLP based	**Optimized using RPART**
Confusion Matrices		
True Positives (TP)	70	668
False Negatives (FN)	1903	1305
True Negatives (TN)	22919	22975
False Positives (FP)	231	175
Statistical Significances		
Sensitivity	0.04	0.34
Specificity	0.99	0.99
Enrichment Factor (EF)	3.55	44.78
Balanced Accuracy	0.51	0.67
F-measure	0.06	0.47

Table II. Decision trees resulted from employing RPART method on the SBVS results to identify marginal COX-2 ligands

No.	CP[a)]	Training Set Error Rate	10-fold Cross-validated Error Rate
1.	0.0659	0.0216	0.0216
2.	0.0476	0.0177	0.0180
3.	0.0283	0.0163	0.0166
4.	0.0209	0.0155	0.0158
5.[b)]	0.0106	0.0148	0.0151

[a)]Complexity parameter of the decision tree; [b)]The selected decision tree with the lowest training set error rate and the lowest 10-fold cross-validation error rate (see figure 3). No evidence of overfitting was found since the ratio of the 10-fold cross-validation error rate over the training error rate is less than 1.5 (Cappel *et al.*, 2015).

Table III. The *in silico* screening results on some phytochemicals as COX-2 inhibitors

Name	IC_{50} (nM)[a)]	Decision Tree Parameters[b)]							TP or FN[c)]
		ChemPLP score	PLIF bitstring number						
			17	18	68	138	221	242	
Curcumin	79200	-86.1881	1	0	0	0	0	0	FN
Resveratrol	32000	-90.2518	1	0	1	0	0	1	TP
Radicicol	27	-49.8668	0	0	0	0	0	0	FN
Triptolide	40	-67.6514	0	0	0	0	0	0	FN
Tryptanthrin	64	-87.7228	1	0	0	0	0	0	FN
Rutaecarpine	300	-86.6826	1	0	0	0	0	0	FN
Encelin	400	-66.2878	0	0	0	0	0	0	FN
Enhydrin	600	-37.5564	0	0	0	0	0	0	FN
Parthenolide	800	-63.2679	0	0	0	0	0	0	FN

[a)]Ref: (Bento *et al.*, 2014); [b)]See Figure 3 for more explanation; [c)]TP and FN stand for True Positive and False Negative, respectively

Therneau *et al.*, 2015) was used to generate decision trees using ChemPLP scores and PLIF bitstrings descriptors to optimize the SBVS protocol. The best decision tree (Figure 3) resulted in a confusion matrix with EF value of 44.78. The EF value of the optimized SBVS protocol was higher than the $EF_{1\%}$-ChemPLP based SBVS (3.55) and the reference (12.9). Moreover the predictive ability of the optimized protocol was statistically better in confidence level of 95% compared to the $EF_{1\%}$-ChemPLP based SBVS (p-value < 0.05) using McNemar's test with chi-squared value of 417.23 (Cannon *et al.*, 2007; R Core Team, 2015).

The predictive ability of the optimized protocol was considered as acceptable since it outperformed the reference protocol (Mysinger *et al.*, 2012). By examining the statistical significances (Table I), although the optimized protocol could be used further in prospective campaigns since the EF value was sufficiently high (de Graaf *et al.*, 2011; Istyastono *et al.*, 2015[b]), the sensitivity value was still considered as low (Desaphy *et al.*, 2013). The predictive ability was mainly contributed by the high specificity value. This indicated that if a compound predicted as a COX-2 inhibitor using this optimized *in silico* screening protocol, it would be high likely as COX-2 inhibitor *in vitro*. But, if a compound predicted as a non COX-2 inhibitor, it would still likely be a COX-2 inhibitor *in vitro* since the sensitivity value was low caused by the high number of the false negatives (FN; Table 1). This high number of the FN was the limitation of the optimized SBVS protocol that could be improved to increase the predictive ability of the SBVS protocol by employing some more advanced approaches, for example: (i) employing anchor reactions during molecular docking simulations (Yuniarti *et al.*, 2011) or post-docking pose selection (de Graaf *et al.*, 2011; Istyastono *et al.*, 2015[b]), and/or (ii) employing advanced used of PLIF bitstrings (Desaphy *et al.*, 2013).

Based on Figure 3, the most important descriptor to identify COX-2 inhibitors was the hydrogen bond interaction to ARG499 (previously reported as ARG513 in the older COX-2 crystal structure (Kurumbail *et al.*, 1996)) with the inhibitors as the acceptor (PLIF bitstring #221). This interaction was identified previously as the anchor interaction of COX-2 inhibitors to COX-2 binding pocket (Kurumbail *et al.*, 1996; Wang *et al.*, 2010[a]; Yuniarti *et al.*, 2011). This anchor interaction was identified in the interaction of selective COX-2 inhibitor celecoxib in the COX-2 binding pocket (Wang *et al.*, 2010[a]). Alternatives important interactions identified in this research were: (i) hydrogen bond interaction with the residue as the acceptor, which were to GLN178 (PLIF bitstring # 68; Previously reported as GLN192 in the older COX-2 crystal structure (Kurumbail *et al.*, 1996)), LEU338 (PLIF bitstring #138;

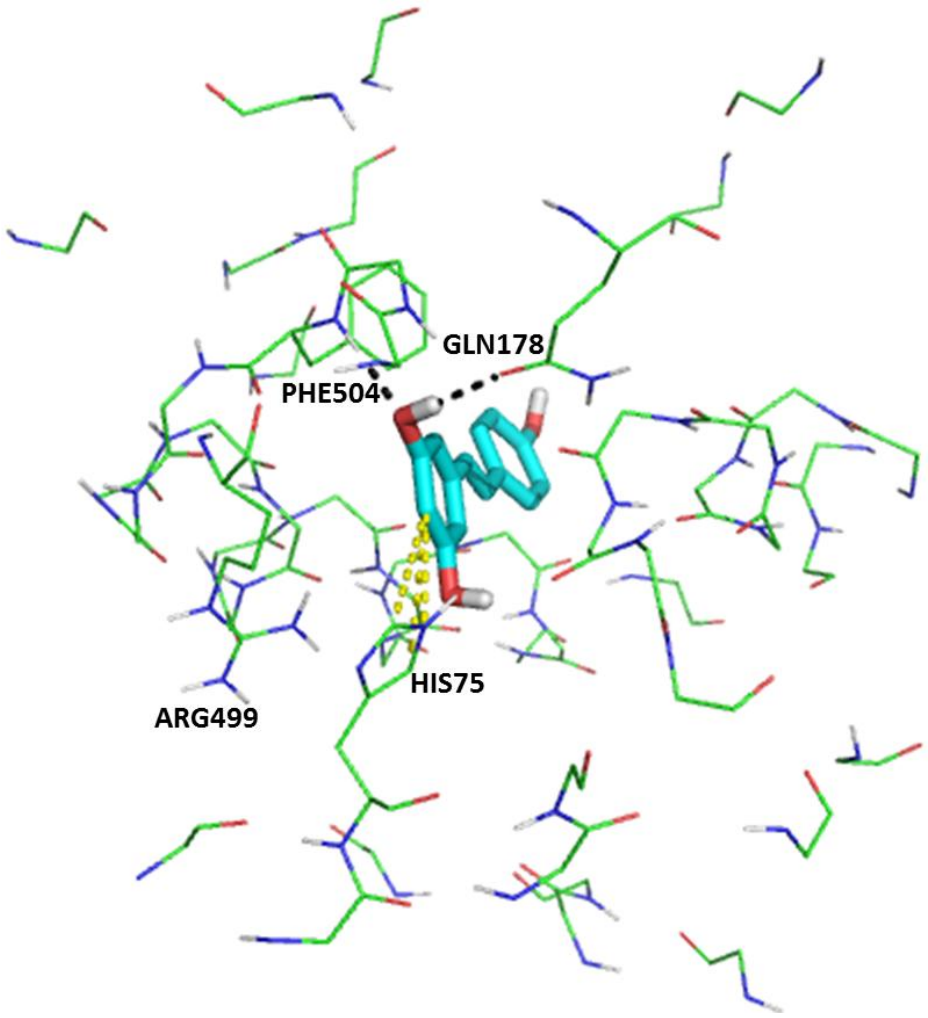

Figure 4. The selected docking pose of resveratrol (sticks mode; carbon atoms in cyan) in COX-2 binding pocket (lines mode; carbon atoms in green). For clarity, (i) only polar hydrogens (in white) are presented, and (ii) only main chain atoms are presented for the binding pocket except for the important residues: HIS75, GLN178, ARG499, and PHE504 (see also Figure 3). Oxygens and nitrogens are colored in red and blue, respectively. Hydrogen bonds and aromatic interactions are presented in black dashed lines and yellow dashed lines, respectively.

Previously reported as LEU352 in the older COX-2 crystal structure (Kurumbail *et al.*, 1996)) or PHE504 (PLIF bitstring #242; Previously reported as PHE518 in the older COX-2 crystal structure (Kurumbail *et al.*, 1996)); (ii) hydrogen bond interaction to HIS75 (PLIF bitstring #18; Previously reported as HIS90 in the older COX-2 crystal structure (Kurumbail *et al.*, 1996)) with the residue as the donor; and (iii) edge-to-face aromatic interaction to HIS75 (PLIF bitstring #17). Celecoxib was also reported having the interaction to GLN178, HIS75 and PHE504 (Wang *et al.*, 2010[a]). The interaction to LEU352 could categorized as novel interaction, but it was not rare in COX-2 binding since very recently several COX-2 inhibitors could proceed further to the clinical trial phase although the compound did not show interaction to the previously known important residues in the COX-2 binding pocket (Wang *et al.*, 2010[a]; Wang *et al.*, 2010[b]). The decision tree

resulted from RPART method was therefore could identify alternative important interactions which in turn could increase the predictive ability of SBVS protocols by decreasing the number of FP and FN. Moreover, the 10-fold cross validation in the construction of decision trees (Table 2) showed that there was no evidence of overfitting of the selected decision tree, and the 1000 times Y-randomization showed that there is no evidence of chance correlation (Cappel *et al.*, 2015; Lim *et al.,* 2009).

Phytochemicals Virtual Screening Employing Optimized Protocol

Nine compounds presented in Figures 1 and 2 were examined using the optimized SBVS protocol (Figure 3 and Table 1). The results are presented in Table 3. Surprisingly, only resveratrol was predicted as a COX-2 inhibitor in this research. It was therefore suggested that beside the protocol should be improved to reduce the number of FN, as described in the previous subsection, the protocol exclusively identified marginal COX-2 inhibitors. This should be verified by testing more representative numbers of other external marginal and potent COX-2 inhibitors.

The docking protocol used here is the same as the docking protocol employed by Mumpuni *et al.* (2015), which re-dock co-crystalized ligand celecoxib to the crystal structure 3LN1 with the root-mean-square deviation (RMSD) value of 0.525 Å. Since the value was less than 2.0 Å (Mumpuni *et al.*, 2015), the selected pose of resveratrol here (Figure 4) could be considered as the right pose. In the visual inspection on the best pose of resveratrol in COX-2 binding pocket (Figure 4) using PyMOL (Lill and Danielson, 2011) and the examination of Figure 3 and Table 3, resveratrol was predicted as COX-2 inhibitor by binding to HIS75 (edge-to-face aromatic interaction), GLN178 (hydrogen bond), and PHE504 (hydrogen bond). Surprisingly, the selected pose for resveratrol did not bind to ARG499. The decision tree (Figure 3) provided alternative interactions in COX-2 ligand binding (Wang *et al.*, 2010[a]; Wang *et al.*, 2010[b]). Since the first branch of the decision tree involved hydrogen bond to ARG499 (Figure 3), employing this as the anchor interaction in the molecular docking simulation (Wang et al., 2010a; Yuniarti et al., 2011) could therefore increase the predictive ability of the SBVS protocol.

CONCLUSIONS

The optimized SBVS protocol employing PLANTS1.2 and PyPLIF followed by RPART method to produce decision tree to identify phytochemicals as COX-2 inhibitors has been retrospectively validated using DUD-E with additional marginal active compounds as the active compounds. The protocol resulted in better predictive ability in COX-2 inhibitors identification compared to the original protocol accompanying the release of DUD-E. However the sensitivity value was still considered as low, which was also indicated by predicting correctly only 1 out of 9 phytochemical as COX-2 inhibitors. The improvement could be achieved by employing hydrogen bond to ARG499 as the anchor interaction during the molecular docking simulations using PLANTS1.2 before the PLIF identification using PyPLIF.

ACKNOWLEDGEMENT

This work was financially supported by the *Penelitian Hibah Bersaing* (No. 010/HB-LIT/III2016) from Directorate of Research and Community Services, Ministry of Research, Technology and Higher Education, the Republic of Indonesia. Dr. Sri Hartati Yuliani, Apt. from Division of Pharmaceutical Technology, Faculty of Pharmacy, Sanata Dharma University is acknowledged for her technical assistance and suggestions in writing this article.

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