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Development of a dimer-based screening system for dimerization inhibitor of HIV-1 protease

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Abstract

An *in vitro* dimer-based screening system (DBSS) for selecting new HIV-1 protease dimerization inhibitor candidates from natural compounds had been established. This system utilizes a fusion between HIV-1 protease and dimer binding domain of AraC protein (proteaseHIV1-AraCDBD) where fluorescence signal will be emitted in the presence of HIV-1 protease inhibitor. However, this screening system had not been evaluated. Therefore, this study was aimed to evaluate it in recombinant *Escherichia coli* culture. The expression of proteaseHIV1-AraCDBD fusion gene was observed for 18 hours. Its crude lysate isolation was done once every 3 hours and analyzed using SDS PAGE. To test the DBSS, darunavir was used as positive control, and *Nigella sativa* extract (JH3) was used as the test compound. The results of SDS PAGE analysis on crude lysates presented a ~24.2 kDa band, which was the predicted size of the proteaseHIV1-AraCDBD fusion protein based on its amino acid sequence. The growth curve and protein expression profiles revealed that the 15 hours was the optimum culture age to be used in the screening system. Darunavir testing in DBSS showed an increase in fluorescence signal compared to the negative control. The same increase in fluorescence signal was also obtained from the JH3 compound test. In conclusion, DBSS could be used as an assay to screen for new HIV-1 protease inhibitors, and the JH3 compound demonstrated the ability to inhibit HIV-1 protease dimerization.

Keywords: dimer based screening system, HIV, protease

Introduction

Today, HIV is still a global problem, especially in developing countries. In 2019, there were around 38 million people living with HIV, 1.7 million new infections, and 690 thousand AIDS-related deaths in the world (UNAIDS, 2020). Even with this urgency, the coverage of antiretroviral therapy (ART) is still low. For example, it only covers 17% of all adult HIV patients (aged 15 years and over) in Indonesia (UNAIDS, 2019).

The antiretroviral therapy (ART) itself, both first-line and second-line therapies, are constrained by the high level of virus mutations that enables the virus to evolve resistance quickly (Cuevas *et al*. 2015). One of the drugs used in second-line therapy is darunavir,

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which belongs to the protease inhibitor (PI) drugs. This drug is relatively expensive, but it has a high genetic barrier. Genetic barrier is the number of mutations required by the virus to obtain resistance. PI drugs like darunavir inhibit the dimerization of HIV-1 protease important for HIV maturation (Cuevas *et al*. 2015, Aoki *et al*. 2018, Wang *et al*. 2012, Lascar&Benn, 2009). Unfortunately, an HIV mutant resistant to darunavir has been found (Zhang *et al*. 2014). This has increased the priority in seeking new protease inhibitors.

Natural compounds could be a cheaper and more readily available alternative to replace darunavir in tropical countries like Indonesia. In this regard, Indonesia has many potential untested natural compounds. However, a selection method that enables us to screen through many natural compounds in a short time (high throughput screening system) and could be done in a low biosafety level is not yet available. Thus, this study was conducted to optimize DBSS for high throughput screening system by using darunavir as model protease inhibitor. DBSS was further applied to test the ability of JH3 (extracted from *N. sativa*) in inhibiting the dimerization of HIV-1 protease. JH3 remained undisclosed in this study since it is currently under consideration for patent.

Materials and methods

The materials and methods used are described as follows.

System overview

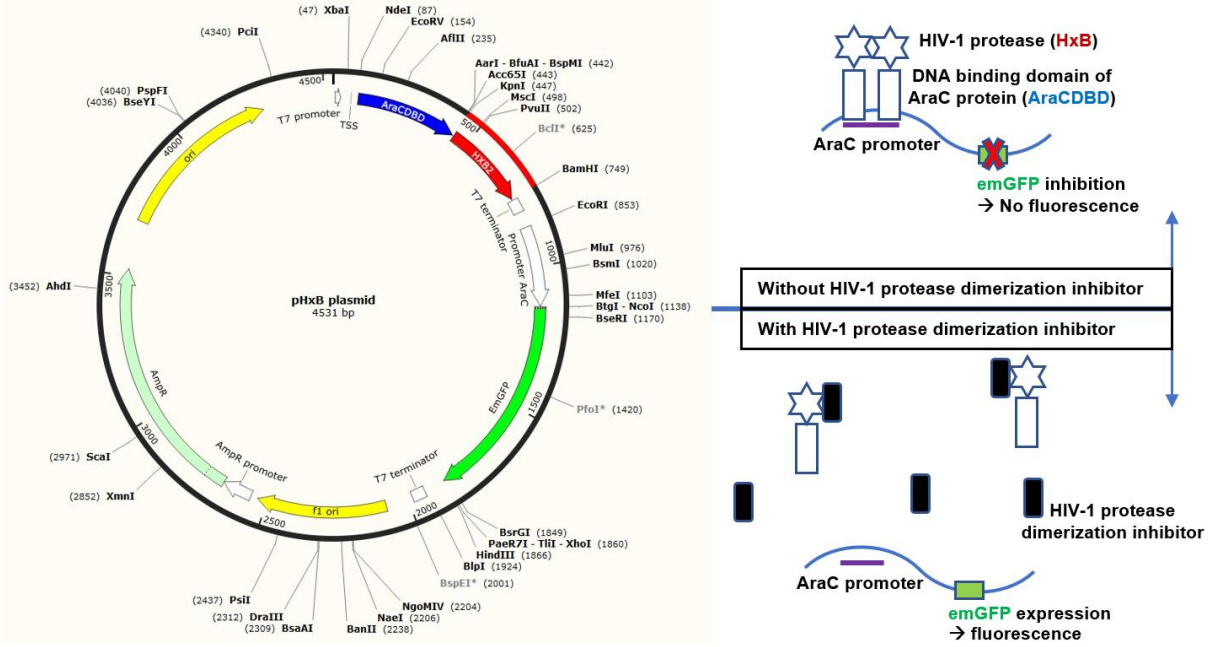

Figure 1. DBSS illustration. Without any presence of protease inhibitor, the system will not emit any fluorescence signal. The plasmid is identical with the one used by Fibriani *et al*. (2018). Origin of replication is marked by yellow, promoters and terminators by white, AraCDBD gene by blue, HxB (HIV protease) gene by red, emGFP gene by green, and AmpR (ampicillin-resistant) gene by light green.

Dimer-based screening system (DBSS) is intended to be used for screening natural compounds for its ability to inhibit the dimerization of HIV-1 protease. This system does not occur naturally, thus it is necessary to design and develop such system in scientific studies. The plasmid used here is identical with the plasmid from the previous study by Fibriani *et al*. 2018. The multi cloning site of the plasmid is located between NdeI and BamHI where HIV-1

protease gene (HxB) is fused with the DNA binding domain of AraC protein (AraCDBD) as shown in Figure 1. DNA binding domain from AraC protein (AraCDBD) in its dimeric state could bind to the AraC promoter sequence which controls the expression of a reporter gene to repress its transcription. Dimerization inhibition of the HIV-1 protease attached to the AraCDBD would cause transcription of the reporter gene to occur. The reporter gene used was the Emerald Green Fluorescence Protein (EmGFP) gene in pRSET plasmid that will emit fluorescence signal when transcribed. The fusion gene sequence (proteaseHIV1-AraCDBD) was placed under T7 promoter. Meanwhile, emGFP gene was placed under AraC promoter downstream of the fusion gene (Okada *et al*. 2007; Fibriani *et al*. 2018). This plasmid will be referred to as pHxB. The positive control plasmid was made by using the same system but without inserting the HIV-1 protease gene. It was expected that without the presence of HIV-1 protease, the expression of emGFP gene would occur unhindered. This plasmid will be referred to as pDBD. This system was run in *E. coli* BL21(DE3) as the host cell. where darunavir was used as positive control.

Plasmids and growth condition

Plasmid construction, including HIV-1 protease gene (HxB) selection step, was commenced by our previous study (Fibriani *et al*. 2018). Here, the practical application of the plasmid was done in *E. coli* BL21 (DE3) (Thermo Fischer, USA). Upon transformation, the bacteria were grown in LB (Luria-Bertani) medium containing 200 µg/mL ampicillin (Phapros, Indonesia). The presence of proteaseHIV1-AraCDBD fusion gene in pHxB plasmid and DNA binding domain gene from AraC protein (AraCDBD) in pDBD was confirmed using polymerase chain reaction (Bio-Rad, Singapore) and DNA sequencing (Supplementary Figure 1 and Supplementary Figure 2). The primers used for PCR were 5'-CTGGAAAGGATCCATGGATAATCGGGTACG-3' (forward primer) and 5'-CATAGCACCATGGTTCATACTCCCGCCATTCAG-3' (reverse primer). Restriction enzyme used for cloning purpose of the fusion gene was NdeI and BamHI. Plasmids and amplicons were analyzed using gel electrophoresis in 1% agarose (Sigma-Aldrich, Singapore) at 100 V for 25 minutes. DNA sequencing of the proteaseHIV1-AraCDBD fusion gene and AraCDBD gene was carried out using T7 promoter and T7 terminator primers at Macrogen Inc., Korea.

Protein analysis

Protein analyses were performed on cultures of *E. coli* BL21(DE3) transformant containing pHxB and *E. coli* BL21(DE3) without plasmid as a control. After going through two subculturing stages in LB (Luria-Bertani) broth with 200 µg/mL ampicillin, the bacteria were grown in 10 mL LB broth in 50 mL centrifuge tubes (NEST, China). One culture tube was taken every three hours, and its optical density was measured using spectrophotometer (Shimadzu, Japan). When the optical density measured was greater than 1, the culture was diluted ten times, and optical density measurement was repeated. Measurements were performed from 0 to 18 hours. At each observation point, protein sampling was carried out. The volume of the sample taken for protein analysis was based on Equation 1:

$$Volume\ taken\ (mL) = \frac{2.5\,x\,1}{measured\ OD_{600}} \quad (1)$$

Equation 1 meant that for each OD_{600} of 2.5, 1 mL of culture was taken. The culture samples were precipitated by centrifugation at 14,000×g for 1 minute, followed by weighing of the pellet. For every 0.02 gram of pellet obtained, 100 µL of SDS-PAGE sample buffer were added. The mixture was vortexed, then boiled at 100°C for 10 minutes. To prevent

protein degradation, phenylmethylsulphonyl fluoride (Sigma-Aldrich, Singapore) was added to the final concentration of 1 mM. Samples were stored in the freezer at -20°C avoiding light. The frozen samples were briefly incubated in boiling water before further pipetting. For each sample, 10 µL was analyzed using SDS-PAGE (12% acrylamide; 1 mm thickness [Bio-Rad, Singapore]) at 100V for 120 minutes.

Darunavir and JH3 inhibitory activity test

The transformed bacteria used in the fluorescence measurement were very sensitive to storage at 4°C. It is recommended to streak bacteria directly from -80ºC glycerol stock onto solid LB medium, incubate for 16 hours, then store at 4°C for not more than 24 hours before use.

Single colonies of *E. coli* BL21(DE3) without plasmid, pHxB transformant, and pDBD transformant from solid media were inoculated into 10 mL LB broths with 200 µg/mL ampicillin. The cultures were incubated in a shaker incubator at 37°C and 200 rpm for 16 hours without IPTG due to leaky plasmid expression. Then 10 µL of the cultures were transferred into 96-well LUMITRAC™ white microplate (Greiner Bio-One, Indonesia) where each well contained 85 µL LB broth with 200 µg/mL ampicillin and 5 µL JH3 or darunavir (Prezista, USA) dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Singapore). pHxB transformant culture in 5% DMSO without the test compound was used as the negative control, and pDBD transformant culture with 5% DMSO was used as the positive control. Accordingly, LB broth containing 200 µg/mL ampicillin with/without DMSO/JH3 was used as blank. Darunavir tests were performed at concentrations of 5, 10, 15, 20, and 25 ppm while JH3 tests were carried out at concentrations of 1, 5, and 10 ppm. The microplate was then incubated in a shaking incubator at 37°C, 200 rpm for 14-16 hours.

The fluorescence intensity of the culture was measured using Glomax™ Explorer (Promega USA), with excitation at 475 nm and emission at 500-550 nm. The proteins were not harvested from the cells since it will complicate the detection process, thus defeating the purpose of developing a high throughput screening system. Optical density of the culture was measured using BioRad™ xMark plate spectrophotometer (Bio-Rad, USA) at 600 nm using a 96-well clear microplate (Greiner Bio-One Indonesia). The fluorescence data obtained was then normalized to its blank and optical density using Equation 2 (Okada *et al*., 2007):

$$Relative\ flourescence\ intensity = \frac{(E_{535}\ sample - E_{535}\ medium)}{(OD_{600}\ sample - OD_{600}\ medium)} \qquad (2)$$

Where E_{535} is the fluorescence emission at 535 nm, and OD_{600} is the optical density at 600 nm. The normalized fluorescence intensity is an arbitrary unit (a.u.)

Statistical analysis

Comparative analysis of optical densities between *E. coli* BL21(DE3) culture without plasmid and pHxB transformant at each measurement point from the growth curve was performed using the Mann-Whitney U test. On the other hand, the comparison of the normalized fluorescence intensity between control, between treatment, and between control-treatment groups were carried out using the Mann-Whitney U test after its Shapiro-Wilk test stated that most of the data were not normally distributed.

Fusion protein 3D modeling and docking

Fusion protein 3D modeling was carried out using I-TASSER (Zhang 2009, Roy *et al*. 2012, Yang & Zhang 2015). To find out how exactly darunavir fit in the predicted fusion protein binding site, molecular docking was carried out using Autodock Vina (Trott & Olson 2010). Darunavir was blindly docked to the proteaseHIV1-AraCDBD fusion protein

monomer models by including the entire fusion protein structure within the grid. The docking process was only carried out for darunavir because there was no available structure for JH3 yet. The docking results were visualized using the help of ICM Browser (Abagyan *et al*. 1994, Raush *et al*. 2009).

Results

Growth curve of the *E. coli* with and without pHxB plasmid is shown in Figure 2.

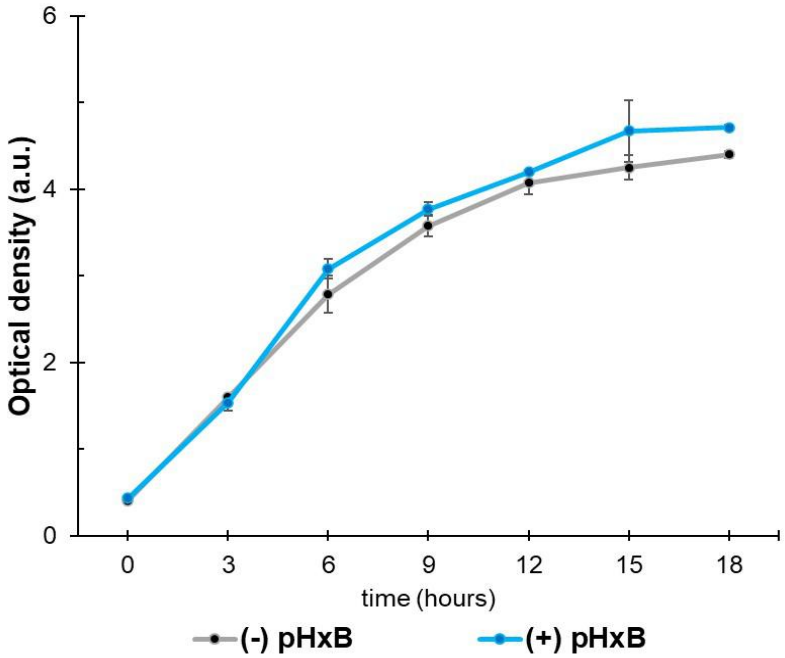

Figure 2. Growth curve of *E. coli* BL21 (DE3) with and without pHxB plasmid. The latter was used as negative control. Transformant colony is referred to as "(+) pHxB", meanwhile colony without plasmid is referred to as "(-) pHxB". Data represent the means ± s.e.m. (n = 2). It could be concluded that the pHxB transformant was able to grow in the given condition.

SDS-PAGE of the crude cell lysates of *E. coli* BL21(DE3) with and without pHxB plasmid is shown in Figure 3. There was no significant difference in optical density ($p > 0.05$) between *E. coli* BL21(DE3) with and without pHxB plasmid on the growth curve (Figure 2). Based on the SDS PAGE profile, the total protein produced by *E. coli* BL21 (DE3) with and without pHxB plasmid at each measurement point was identical except for the ~24.2 kDa band (shown in red box) which was the predicted size of the proteaseHIV1-AraCDBD fusion protein based on *in silico* approximation of its amino acid sequence (Figure 3). The red box highlighted the suspected fusion protein band. It could be concluded that the proteaseHIV1-AraCDBD had been produced as early as 3 hours after inoculation.

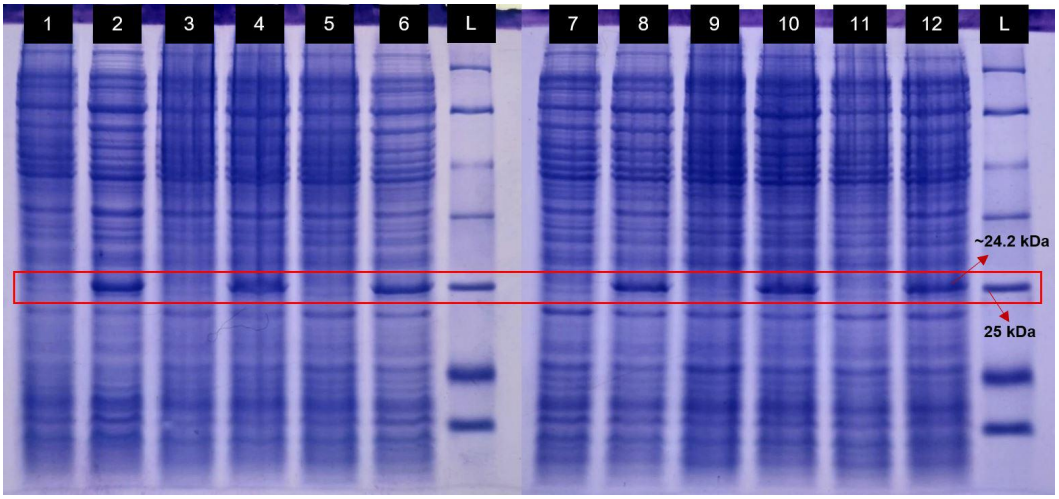

Figure 3. SDS-PAGE of crude lysates from *E. coli* BL21(DE3) with and without pHxB plasmid. The latter was used as negative control. Sequentially from number 1 are: (1) 3-hours culture of BL21(DE3); (2) 3-hours culture of pHxB transformant; (3) 6-hours culture of BL21(DE3); (4) 6-hours culture of pHxB transformant; (5) 9-hours culture of BL21(DE3); (6) 9-hours culture of pHxB transformant; (L) Ladder peqGOLD marker protein I, (7) 12-hours culture of BL21(DE3); (8) 12-hours culture of pHxB transformant; (9) 15-hours culture of BL21(DE3); (10) 15-hours culture of pHxB transformant; (11) 18-hours culture of BL21(DE3); (12) 18-hours culture of pHxB transformant.

The result of the darunavir inhibitory activity test is shown in Figure 4.

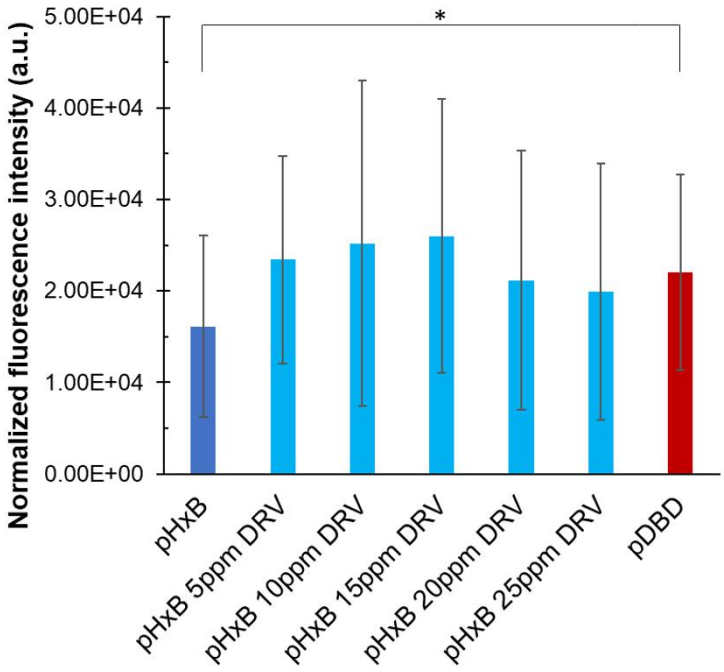

Figure 4. Comparison of the normalized average fluorescence intensity from pHxB transformant at different concentrations of darunavir (DRV). pHxB transformant without darunavir (pHxB) was used as negative control and pDBD without darunavir (pDBD) was used as positive control. Data represent the means ± s.e.m. (n = 7). It could be concluded that darunavir insignificantly inhibited the dimerization of HIV-1 protease.

Figure 4 showed that the normalized average fluorescence intensity of pHxB transformant with darunavir at various concentrations was higher than the fluorescence intensity of pHxB transformant without darunavir (pHxB). There was a significant difference between the negative control (pHxB) and the positive control (pDBD) ($p \leq 0.05$). Meanwhile, no darunavir concentration resulted in a significant increase in fluorescence intensity ($p > 0.05$).

The result of JH3 inhibitory activity test is shown in Figure 5. Fluorescence intensity measurements in Figure 5 showed that 1 ppm of JH3 resulted in a significant increase compared to the negative control ($p \leq 0.05$). Meanwhile, 5 and 10 ppm of JH3 did not produce the same significant increase ($p > 0.05$).

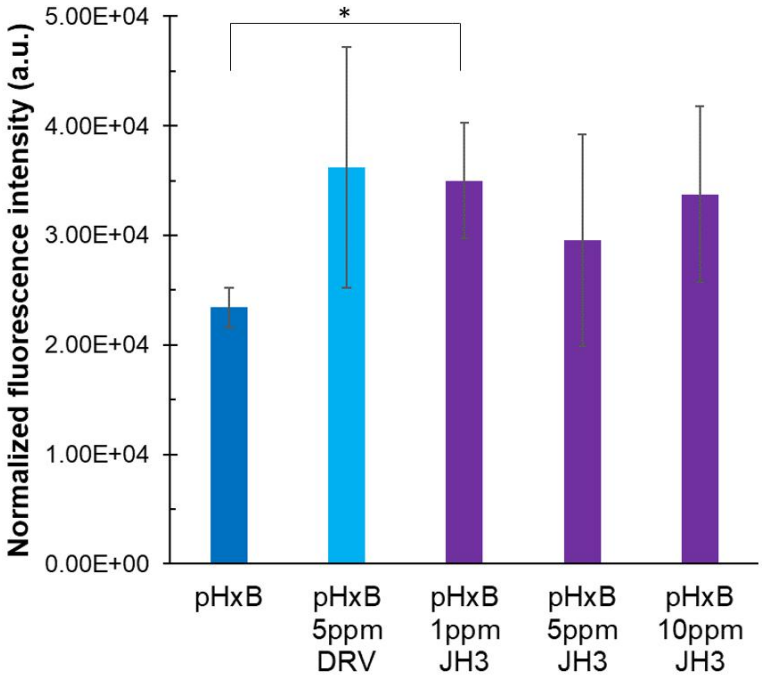

Figure 5. Comparison of the normalized average fluorescence intensity from pHxB transformant at different concentrations of JH3. pHxB transformant without darunavir (pHxB) was used as negative control. Data represent the means ± s.e.m. (n = 6). It could be concluded that JH3 was able to inhibit the dimerization of HIV-1 protease.

In order to find out the darunavir binding site on the fusion protein structure, the fusion protein monomer was modeled using I-Tasser and blindly docked to darunavir. Five most possible models were used in this experiment because the exact structure of the fusion protein monomer was unknown. Meanwhile, JH3 docking was not performed due to the lack of any available structure. The results are shown in Figure 6.

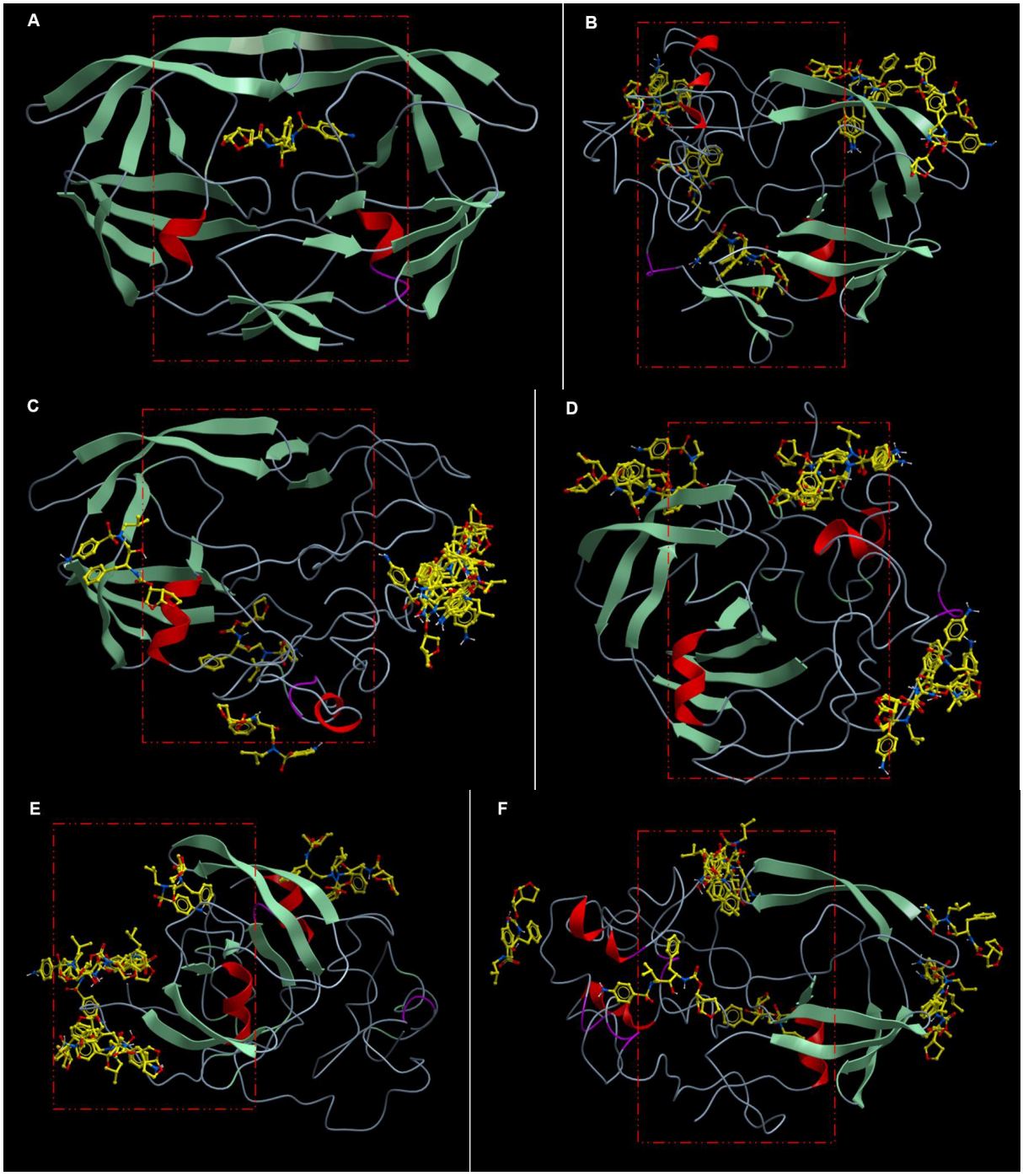

Figure 6. Wild type HIV-1 protease dimer and proteaseHIV1-AraCDBD monomer 3D structure and docking models. Sequentially from A are: (A) wild type HIV-1 protease dimer (PDB: 4LL3) bound to darunavir; (B) proteaseHIV1-AraCDBD model 1 blindly docked by darunavir; (C) proteaseHIV1-AraCDBD model 2 blindly docked by darunavir; (D) proteaseHIV1-AraCDBD model 3 blindly docked by darunavir; (E) proteaseHIV1-AraCDBD model 4 blindly docked by darunavir; (F) proteaseHIV1-AraCDBD model 5 blindly docked by darunavir. The red box highlighted the dimerization side of the fusion protein. Darunavir is colored in yellow.

Discussion

There was no significant difference in optical density ($p > 0.05$) between *E. coli* BL21(DE3) with and without pHxB plasmid at all measurement points in Figure 2. This data showed that the fusion protein expression by the pHxB transformant did not show any sign of apparent toxic effect that might inhibit bacterial growth.

The fusion protein expressed was visible as one thick band that was slightly smaller than 25 kDa (shown in a red box). Since the mentioned band only appeared in pHxB colony samples, that protein band was expected to be the 24.2 kDa-sized proteaseHIV1-AraCDBD monomer. The thickness of those protein bands did not differ much between measurement points. However, the oldest cultures with the thickest protein band were the 15- and 18-hours cultures. Therefore, the fluorescence intensity was measured from the 15-hours old culture (Figure 3).

In this system, recombinant protein expression was not done through IPTG induction because of the expression leakage. LacUV5's operon system integrated with the T7 promoter system discussed intensively by Studier & Moffatt (1986), and Studier *et al*. (1990) highlighted the importance of IPTG/lactose addition to inhibit LacI repressor binding and induce T7 RNA polymerase expression. However, expression leakage in this system was suspected to be caused by the LacI repressor's ineffective binding to the LacO sites.

Both darunavir and JH3 addition resulted in increased normalized average fluorescence intensity. Figure 4 showed that the fluorescence intensity of pHxB transformant with darunavir at various concentrations was insignificantly higher than the negative control. However insignificant, this fluorescence intensity increase was still an indication that darunavir could inhibit the dimerization of the protease. On the other hand, JH3 exhibited the same property. Figure 5 showed that all JH3 concentrations increased the normalized intensity with a significant increase observed at 1 ppm ($p \leq 0.05$). Although the fluorescence intensity increase caused by JH3 had not exceeded darunavir's highest fluorescence at 5 ppm, JH3's potential as an HIV protease dimerization inhibitor could be confirmed.

The 3D protein modeling and docking results in Figure 6 might be able to explain the high deviation standard observed in the darunavir and JH3 inhibitory activity tests. Of the five models produced, model 1, 2, 3, and 5 had its dimerization side (the area inside the red box) structurally hindered. If they were able to form dimers, it would be difficult for darunavir/JH3 to block its dimer formation. It was unknown which model actually existed in the cell. If there were more than one conformation, the ratio of each conformation was unknown. The fluctuation might have occurred because each cell had considerably different ratio of these conformations.

In conclusion, DBSS could be used to test the dimerization inhibitory activity of darunavir and JH3 towards HIV-1 protease, where both compounds were able to show such inhibitory activity. In the future, it is important to obtain a more reliable result especially with lower deviation standard. An assay based on different incubation durations could be done to find out the most stable fluorescence emission point. Furthermore, the exact structure of the fusion protein needs to be elucidated to clarify the darunavir/JH3 binding model.

Conflict of interest

The authors state no conflict of interest in this manuscript.

Acknowledgment

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Author contributions

YMS extracted JH3. IDAPD collected *in vitro* data. IDAPD and RA performed *in silico* analysis. All authors designed the experiments and analyzed the data. IDAPD, F and AF wrote the manuscript.

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