MOLECULAR DYNAMIC SIMULATION IN GREEN DRUG DEVELOPMENT

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Abstract

Today, finding new drugs for treatment is much more tedious than finding mechanism of diseases, with the long time and high cost for success marketing new drug, motivation of scientists in the field of medicine is low down. Drug repositioning is considered as a new strategy for designing drug. This "computer-aided drug design" field or the *in silico* study is now widely used in international pharmaceutical institutes and companies in evaluating protein structures, understanding the chemical reagents, or calculating the effect of drug binding... To illustrate the advance of molecular dynamic simulation, two studies in breast cancer were introduced. One study used the techniques in investigating the different between estrogen ligands, while another showed the mechanism of inhibition. These computer-aided drug design techniques not only provide the actual phenomenon in elucidating the dynamic nature of macromolecules and insights into the impact of mutations as well as drug adverse effects, but they also be the best green technological routine, when concern about environment is getting more attention worldwide.

Keyworks: Molecular dynamic simulation, Steered molecular dynamics (SMD), Drug development

Advances in molecular biology give more opportunity to discover the mechanisms of diseases thus develop more effective treatment. One example is that cancer used to defined as a development of abnormal cells in different tissues and organs with only surgery, chemotherapy and radiotherapy as treating methods; it now is known that the mechanism of cancer is very complicated with the participant of many molecular factors, such as genes, proteins, cellular responses. And based on those mechanisms, now people find new ways to treat cancer using hormone-therapy, gene-therapy or immunotherapy… which cause less and milder side-effects for patients than others (1-3). On other aspect, finding new drugs for treatment is much more tedious than finding mechanism of diseases. The traditional approach in drug development, or the *de novo* drug development process, takes 13 to 15 years and about 1 billion for one drug brought to the market (4, 5). The rate of success marketing new drug is now decreasing and the cost is continue increasing which low down the research and development motivation of scientists in the field of medicine (6). In additional, this technology could harm people environment by many experiments without careful looked. Most of the time, the drugs are developed based on the chemicals or biological agents, which can kill and affect one or other species. For complex disease like cancer, drug development becomes more crucial and challenging.

The drug candidates have to go through a strictly process for several trials before it is approved to use on human. After discovering in the laboratory, the candidates must provide safety evidences which are tested on human cells or animal models. Then, they have to register for the clinical trials on human including 3 phases to test the toxic property, the effective treatment, and the side-effect, which is very expensive. In addition, some diseases caused by microorganisms, such as bacteria, virus, fungi… may hold a resistant property. These microorganisms can change

the structures, so that they can easily escape our immune systems and resist to current drugs (7). Especially, today atmosphere changes dramatically that leads to the out-of-date target for candidate drug when the drug is approved for market. That will cause the commercial drug only having effect for short time or low effect in treating. At that time, efforts need to make to modify the drug for changed target and apply clinical trial testing again. Those are reasons for finding new drug to treat diseases increasingly difficult.

One strategy had been developed recently to overcome the limitation of *de novo* drug development, that will maximize the value of the optional potential of drugs, and minimize the bad effects on the environment. It is called drug repositioning, or drug re-profiling, or drug review process, or treatment modification is the process to identify new indications for drugs (figure 1). Drug repositioning can be a solution as an additional strategy since already approved drugs are generally safe and the risk of failure is decreased. The cost is significantly cheaper than *de novo* and the cycle time for development is also shorter than *de novo* one. This approach is also considered as the green technology in drug development. In the first stage, without the join of any chemicals, animals or reagents that can cause burden to the environment, the molecules are tested *in silico* for its target's effect. This will limit the waste from the biological laboratory, if the molecule is not working.

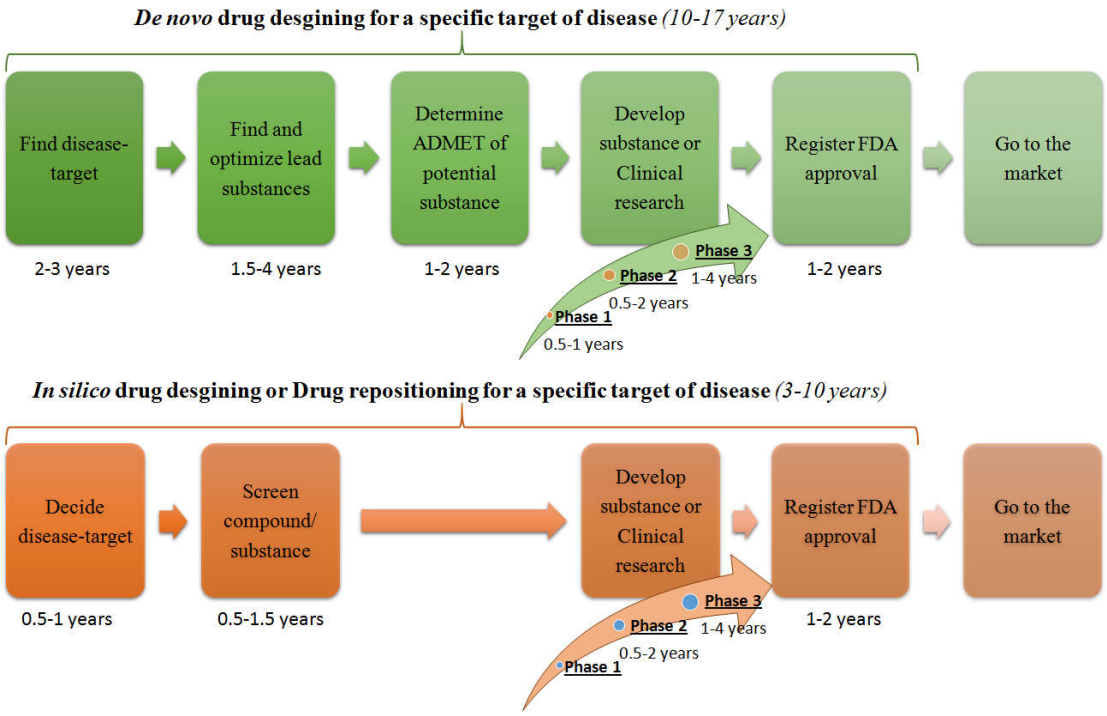

Figure 1: Strategy of *de novo* drug discovery and development was compared with drug repositioning, new strategy for drug design (8).

Drug repositioning not only requires the laboratory as normal, but it also requires knowledge in computational science. With the development of computer science, the drug development has been helped to shorten the time and lower the cost for commercial drugs. "Computer-aided drug design" is named to this field and emphasis the role of computer science in designing drug. This field is now widely used in international pharmaceutical institutes and companies. *In silico* studies can help in evaluating protein structures, understanding the chemical reagents, or calculating the effect of drug binding...

According to Jin and Wong, there are different virtual screening pipeline used and listed in the field of computer-aided drug design (9). These methods are developed based on three bases: drug-oriented, disease-oriented, and treatment-oriented. Among them, drug-oriented basis is the

earliest approach and most popular methods used to repositioning drug. It is simple, fast and easy to use. However, the disease- and treatment-oriented bases are more exactly than the drug oriented basis, because they show the actual data collected when human receive a drug. Human body is complex and one drug does not only affect one but many pathways which have the same targets. This leads to the complex outcome when people using drug for treatment. The methods developed in these bases require knowledge of specialists knowing about medical and science aspects to identify the signature of disease and drug for repositioning. Pharmacogenomics is the field in which people investigate the effect of human genome on their response to drug. Thanks to the next generation sequencing technologies, the genomic data become more available thus our chance to develop effective personalized medicine become more promising.

Target-based method and Molecular dynamic simulation

The target-based method in drug repositioning remains the most popular one so far. Two main ways for this method are docking and reverse docking (figure 2). The docking is defined as a method to predict and calculate the binding score of a ligand to a known 3D structure protein. The highest score will give the predominant binding mode(s) between ligand and protein. This is the routine used in structure-based drug discovery for hit identification and lead optimization (10). Meanwhile, the reverse docking is used for ligand-based drug design, which will dock a small molecule on a pool of protein structures. For the second way, there is one limitation in using this that the databases for the protein structures have not yet known. There are still many proteins with unknown structures and without correct 3D structures, the prediction will be fail. One ligand is considered as a drug candidate will have highest score of binding on target protein, and its scaffold can be the starting point for strategic plan in further design.

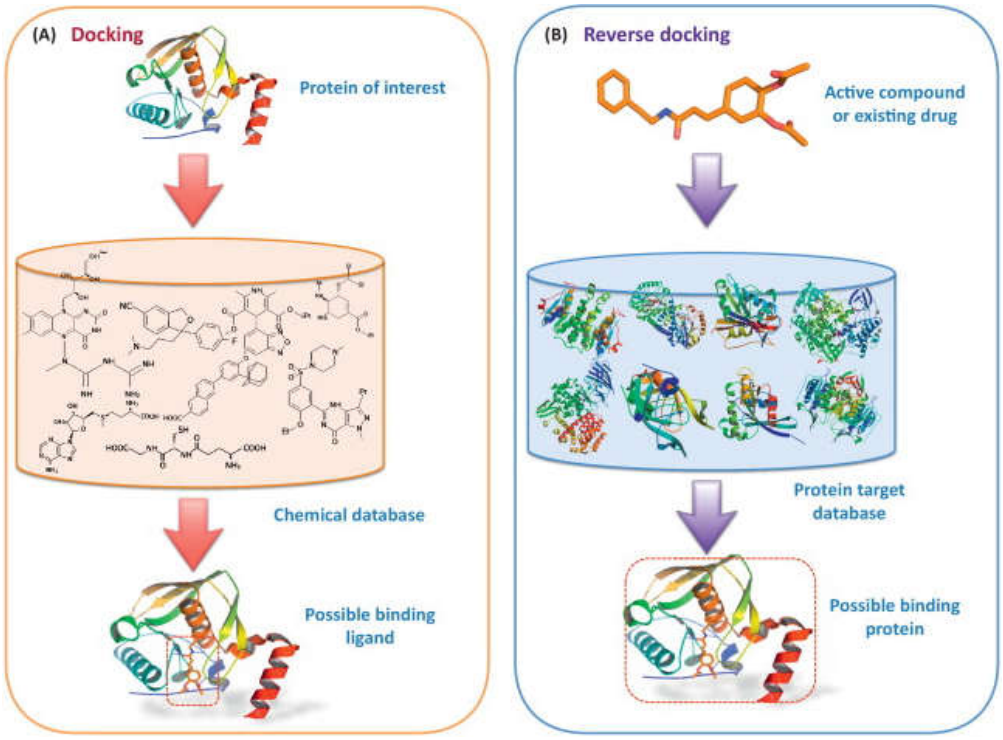

Figure 2: Schematic diagrams of two ways in target-based method in drug repositioning: **(A)** docking of different similar structured chemicals to one target and **(B)** reverse docking of different targeted proteins to one chemical in 'hit' identification (10).

Noticing the advantage of computer-aided drug designs, many chemists and scientists in computer science shifted to bioinformatics field which provide a great support for drug design. Many algorithms were used and developed for estimating binding score between ligand-protein or protein-protein interaction. For conventional docking, lot of software are introduced in website of Virtual Ligand Screening (11). Among them, the most popular software used in docking are DOCK, AutoDock, FlexX, GOLD and GLIDE (12).

These methods have been widely applied in finding and repositioning drugs for new indications from the original one. At first, this method was used widely in quantum chemistry which simulates the reaction between inorganic elements. The simulation will provide the data of

emitted energy of the reaction and apply for creating chemi-energy. When the development of drug got difficulty in discovering new drugs, the scientists tried to reuse the old drug for new diseases, and cheminformatics is used to screen the drug database for drug candidates. At that time, attention in quantum chemical software were switched to molecular docking which allows observation of interactions between drug candidate and macrobiological systems. However, between human body, human cells or tested animals and the inorganic elements, it is very different. And the simulation or prediction in organic molecules is not easy and simple as in reaction between inorganic molecules. These techniques can not show accurate results in a diverse and complex living body. So, these methods still have not gained the trust of the old drug developed science.

Many docking tools calculate the binding force between protein and ligand in the vacuum environment, which does not give correct and exact results as in reality. There is 70% of living body comprised of water and many small ion molecules which can manipulate the force in binding between molecules. In fact, when a protein or ligand is put in water with different ions, such as H^{+} and O^{2+}, the conformation of the protein and ligand will be changed, which will change the binding site and scoring function. This problem will appear when normal docking methods are used to screening the candidate drugs. Another issue is that proteins are flexible molecules while most of docking procedure assumes that they are completely rigid and only drugs are allowed to move during the docking experiment. Without capturing the protein flexibility, it is difficult to gain the correct binding pose and binding energy of drug candidates when we only use docking approach. To overcome this difficulty, the molecular dynamic (MD) simulation was developed as a new strategy for drug design. MD simulation measures the change of confirmation at every femto- to nano-second to explained about the protein fluctuation. This

method can provide the observation closely to the physiological conditions in elucidating the dynamic nature of macromolecules and understand the impact of mutations and adverse effects (13). In the steered molecular dynamic (SMD) simulation, the protein and ligand are put in the emulated living environment including water and ions, while the protein and ligand are at the predominant binding mode. Then, a force is applied on the ligand and it pulls the ligand out of the protein in the emulated environment. This pulling will make space between protein and ligand and allow ions running to the pocket and making linkage bonds between ions, protein and ligand. When the force reaches the magnitude that the ligand is unstable and released from the pocket of protein, the force magnitude is recoded as the binding force between ligand and protein in living environment (figure 3).

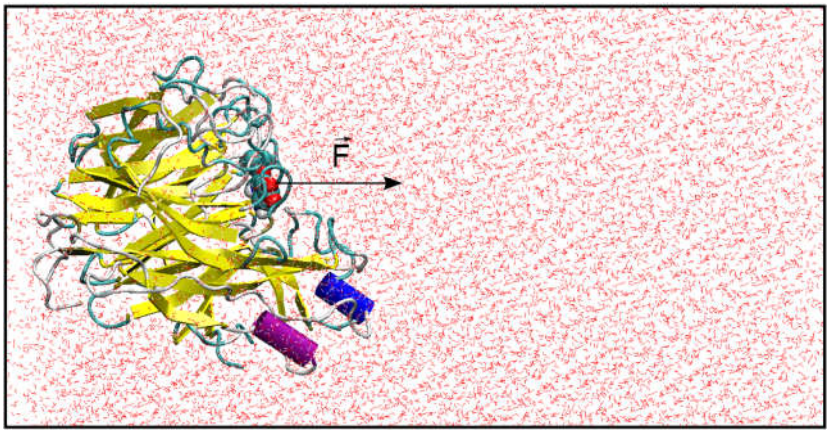

Figure 3: Initial structure for pulling ligand from receptor/protein. The arrow refers to the atom to which the force is applied.

Yet, there are some limitations in molecular dynamic simulation. Most of the limitations are due to the undeveloped algorithm for computer-aided drug design.

Normally, proteins which are used in computer-aided drug design are constructed by NMR, X-ray crystallography and homology modeling. These techniques provide valuable knowledge

about proteins, but it is limited in calculating the binding of proteins. The binding between proteins with other molecules is a very complex process. When a ligand moves to a protein for appropriate binding in an environment, the process is not as one molecule stick to another molecule, it is the binding of ligand with a big macromolecule having mobility. In addition, protein and ligand will possess different conformation in environment with different ion concentration, and each people own his/her own environment. Therefore, the best result given by this protein/environment does not mean same result with others (14). Secondly, the force field for the molecular dynamic simulation is needed to be improved. Now, most of the tools use quantum chemical platform to estimate the binding energy; meanwhile, proteins are macromolecules which are not suitable or the micromolecule-calculating force field. There are many ground works have been developed to improve the force field. CHARMM, AMBER and GROMOS are three most popular force field used in drug design (table 1).

Table 1: Summary of three most force-fields for molecular dynamic simulation (13).

Groundwork	Latest version	Features
GROMOS (1978)	GROMOS05	GROningen MOlecular Simulation package Open source and free software Force fields: n-alkanes, cycloalkanes, isoalkanes, neoalkanes and branched aliphatics Thermodynamic property of small molecules Energy calculation
CHARMM (1983)	CHARMM27	Chemistry at HARvard Molecular Mechanics Rigid molecules simulation Obtain balanced interaction between solute-water, water-water energies Included unsaturated hydrocarbons Compatible for proteins, nucleic acids, lipids and many other small molecules
AMBER (2002)	AMBER99sb	Assisted Model Building with Energy Refinement Polar hydrogen bonds and torsion parameters Better balanced secondary structure elements of protein Better hydrogen bonding and better accuracy

Another limitation is the time of simulation. Most of the simulation tools use platform of molecular dynamic simulation for quantum chemistry which measure the movement and binding in a level of billionths of a second. In fact, the macromolecules change their movement and binding activity slower than the micromolecules in millionths of a second. This limitation does not affect the result, but it makes the time for simulation longer and requires a tremendous computing resource (13).

SMD simulation studies in drug designing for breast cancer

Breast cancer is the top cancer in women both in the developed and developing countries. Not only women get this type of cancer, men can also get breast cancer. Followed the report of American Cancer Society, there are about 2.400 new cases are expected in men among total 235.000 cases in America (15). And each year, nearly 1.7 million new cases of breast cancer are diagnosed worldwide (16). In low- and middle-income countries, where the medical service is still limited, the breast cancer is diagnosed in very late stages (15). And the risk is still high, which will decrease the opportunity of treatment for late stage. It is needed to have more effective anticancer drugs for breast cancer.

Agonist ligands and antagonist ligand: Difference in dissociation from estrogen receptors

The estrogen receptors (ERs), soluble intracellular proteins, are members of the nuclear receptor superfamily. This type of receptor act as ligand-induced transcription factors in the target cells (17). The nuclear receptor superfamily in human consists of 48 proteins including ERs, progesterone (PR), mineralocorticoid (MR), androgen (AR), glucocorticoid (GR) or steroid receptors (SR). Many researches had proved the effect of ER signaling to human diseases, especially to the breast cancer, this receptor is considered as the drug target for breast cancer treatment (18). These drugs act as antagonist to inhibit the action of ERs, antiestrogens, for patients who exhibit ER positive breast cancer (19). Tamoxifen was the first selective ER modulator to be used in clinical treatment of breast cancer and it is still used today (20). However, not all patients have good response to this type of therapy and long-term treatment gives severe adverse consequences, thus there is a need to find new selective pharmaceuticals (21). It is very important to map out and understand the action mechanism of ER, while the ligand binding mechanism on a molecular is still unknown. By using molecular dynamic tools, Burendahl and colleagues had used different structures of ERs and ligands to investigate the

binding and unbinding mechanism of ERs (22). This information provides the insight explanation to specific binding ligands and raises the opportunity for drug design based on selectivity and dissociation time. In their research, 17β-estradiol (EST) and genistein (GEN) were used as the agonist ligands and 4-hydroxytamoxyfen (OHT) as the antagonist ligand. Similarly, different structure of human, mouse and rat ER subtypes were chosen as the receptors on different ligands (table 2). During their work, not only SMD simulation had been used, another tool called random acceleration molecular dynamics (RAMD) also be used to help in finding the possible unbinding pathways of ligand from receptor. This RAMD is very useful together with SMD simulation. While SMD simulation can calculate the force parameter for extracting ligand, the RAMD shows different pathways for the calculation. This will save time for SMD in finding the unbinding pathways which will take long time by its random simulation.

Table 2: Estrogen receptors (ERs) and ligands were used in Burendahl's experiment. EST: 17β-estradiol, GEN: genistein, OHT: 4-hydroxytamoxyfen, -: not mentioned.

PDB	Protein	Ligand	Cofactor peptide	Added Crystal water	Missing residues
1GWR	Mouse ERα chain A	EST	Transcription Intermediate Factor 2 box 3	26	332-334, 462-464
2J7X	Rat ERβ	EST	Nuclear Coactivator-5	26	239-241, 369-374
1X7R	Human ERα	GEN	Steroid receptor coactivator-3	115	331-337
1X7J	Human ERβ	GEN	Steroid receptor coactivator-1	55	411-420
3ERT	ERα	OHT	-	-	411-422, 480-482

As the result, the agonists or selective ER modulators can dissociation from the receptor through seven different unbinding pathways, while it is required larger force and conformation changes

for antagonist dissociated from the receptor (22). Each pathway was then investigated by SMD simulation from the force field, and the maximal force field (F_{max}) was extracted to show the bottleneck of the unbinding trajectory. The lower the F_{max} during time; the easier the unbinding of the ligands from the ERs. Together providing the F_{max}, the SMD simulation also analyzed the nature of amino acids along the pathway in term of polarity, length, width, and identified the important amino acids. As the result, the unbinding pathways which have short pathway length are favorable in most of the systems, so that the ligands do not to travel so far with the receptor to unbind. The SMD simulation also showed the interaction of ligand with other 20 amino acids and at least one of them is polar amino acid during the leaving pathway. This explained for the favorable pathways in most of ligand-receptor systems. Although two methods RAMD and SMD are similar, the advantage of SMD simulation over the RAMD includes the observation along the entire pathway, the timescale of unbinding varying from 0.5 to 1 ns, more hydrogen bond interaction generated, lager events of interaction, and gentler than the RAMD.

Burendahl and colleagues have explained the selectivity of GEN on ERs and the strong binding of antagonist OHT to ERs. These clues will help in designing drug where both the selectivity and time of dissociation are addressed.

Case study in inhibitory mechanism of azole compounds against human aromatase

In another study of breast cancer, the mammalian cytochrome P450s (CYPs) are a diverse group of enzymes that catalyze various versatile reactions in the cell (23). These CYPs involve in the synthesis and metabolism of steroid hormones which play a major role in development and progression of such breast cancer and prostate cancer. Human aromatase CYP19A1 is the only known enzyme that specifically catalyzes the biosynthesis estrogens from androgens (24, 25). In

breast cancer, the improper action of aromatase will cause the increasing level of estrogens, which will lead to abnormal cellular proliferation (26). Due to its role in estrogen biosynthesis, CYP19A1 becomes the major frontline therapy in the treatment of estrogen-dependent breast cancer in post-menopausal women (27). Several aromatase inhibitors have been used in clinical treatment, such as exemestane, anastrozole, letrozole (LTZ) and become effective strategy to combat breast cancer (28, 29). Some inhibitors are competitive inhibitors of aromatase activity, while others act as non-competitive irreversible suicide substrates and inactive the current enzymes (27). However, there are still side effects of these drugs driven the researchers try to find new aromatase inhibitors.

Despite the efforts to unveil the catalytic mechanism and interaction of aromatase with ligands, an intriguing question how substrate/ inhibitor enters into and leaves from the buried active site of aromatase is still lacking. This issue is very important, because the ligand channel will affect the recognition and activity of the ligand on enzyme. Meanwhile, the unbinding pathway serves as the peripheral binding site, which is essential to block the product releasing, a new strategy for drug designation. For those reasons, J. Cai and his colleagues investigated egress pathways LTZ and imazalil (IMZ), an azole fungicide and reported as an inhibitor to aromatase (30), from aromatase CYP19A1 by using both RAMD and SMD simulation method (31). In additional, they also used MM/BPSA method to calculate the binding affinity of these ligands to enzyme for insight mechanism.

As the results, they provided evidence on binding position and affinity between ligands and enzyme firstly (31). The LTZ and CYP19A1 had a uniform binding mode, in which one of the triazole nitrogen atom point to the heme iron and had potential to form a coordinate bond with the iron. While the IMZ (*R*-IMZ and *S*-IMZ) had four different binding modes with CYP19A1.

By MD simulation, it is shown that both *R*-IMZ and *S*-IMZ are less stable within in the active site of aromatase which accounts for weak inhibitory activity. The imidazole nitrogen atom is shifted a little away from iron and led to the increasing distance between two atoms.

Several unbinding pathways of LTZ and *R*-IMZ from the CYP19A1 had been found using RAMD method. The SMD simulation had been used to calculate the maximal force peak of different pathways in each aromatase-inhibitor system. As the results, it is clearly stated that the LTZ and the IMZ will leave the aromatase through different paths, and the IMZ is easier to escape from the active site than LTZ. Certain residues played an important role in the inhibitor dissociation, it is called gating mechanism of pathway (31). Side chain of Phe221 is important in the leaving path of LTZ unbinding aromatase. It is located at the entrance of path and a gatekeeper for unbinding regulation. This Phe221 underwent a rotation to leave sufficient space for the inhibitor to pass, together with the displacement of Gln225, Ser478, and His480. In egress pathway of IMZ, the side chain of Phe134 pointed to the inside of the binding pocket, occupied part of the channel space and then prevented the ligand from leaving the site. This Phe134 is an important factor in stabilizing the binding of ligand to enzyme.

The selection of ligand channels of aromatase appeared to be ligand-dependent, which is proved in the paper with two azole compounds exhibited significantly different inhibitory activities toward aromatase. The work of Cai and others demonstrated the multiple unbinding pathways co-exist in aromatase, and some pathways are favorable. Together with the gating molecules, these evidences can assist in the understanding of the mechanism of action of the azole inhibitors and help in designing new inhibitors for aromatase.

Conclusion

The increase in the population and disease types has created a large demand for effective drug discovery. The genetic variations between people lead to the difference in the drug response, therefore personalized medicine began important field in drug discovery process. It is impossible for screening every compound for each individual variant in laboratory. Hi-technology is new field getting more interesting from scientists to help them in this laborious process. However, together with the concern about the environment, the technologies are judging in their green-side. Thus, bioinformatics will have an important role in future drug discovery to reduce the cost, time, labor and pollution. The evolution of computational methods is increasing with modeling, docking, and MD simulation. The molecular dynamic method can provide the actual phenomenon in elucidating the dynamic nature of macromolecules and insights into the impact of mutations as well as drug adverse effects. Although, there are challenges in the improvement of algorithms for structural bioinformatics dealing with the connection between big data in human genome to drug targets, *in silico* approach including molecular dynamics simulation will be the best green technological routine for drug development and repositioning in genomic era.

Reference

1. Huang C, Li M, Chen C, & Yao Q (2008) Small interfering RNA therapy in cancer: mechanism, potential targets, and clinical applications. *Expert Opinion on Therapeutic Targets* 12:9.

2. Lobo RA (2016) Hormone-replacement therapy: current thinkin. *Nature Reviews Endocrinology* 13.

3. Swart M, Verbrugge I, & Beltman JB (2016) Combination approaches with immune-checkpoint blockage in cancer therapy. *Frontiers in Oncology* 6.

4. DiMasi JA, Hansen RW, & Grabowski HG (2013) The price of innovation: new estimates of drug development costs. *Journal of Health Economics* 22:35.

5. Adams CP & Brantner VV (2009) Spending on new drug development. *Journal of Health Economics* 19(12):130.

6. Daniel GW*, et al.* (2015) Improving pharmaceutical innovation by building a more comprehensive database on drug development and use. *Health Affair* 34:9.

7. Wang G, Zhao N, Berkhout B, & Das AT (2016) CRISPR-Cas9 can inhibit HIV-1 replication but NHEJ repair facilitates virus escape. *Molecular Therapy* 24(5):522.

8. Coma M (2014) Drug repositioning. (ANAXOMICS Systems Biology Solutions).

9. Jin G & Wong STC (2014) Toward better drug repositioning: prioritizing and integrating existing methods into efficient pipelines. *Drug Discovery Today* 1296.

10. Zheng M*, et al.* (2013) Computational methods for drug design and discovery: focus on China. *Trends in Pharmacological Sciences* 34:11.

11. Screening VL (2016) In silico drug design: some concepts and tools. (Virtual Ligand Screening).

12. Alonso Hn, Bliznyuk AA, & Gready JE (2006) Combining docking and molecular dynamic simulation in drug design. *Medicinal Research Reviews* 26:38.

13. Pasupati S & C. GPD (2015) Molecular dynamics: New frontier in personalized medicine. *Advances in Protein Chemistry and Structural Biology* 102:44.

14. Wandzik I (2005) Current molecular docking tools and comparisons there of. *Communications in Mathematical and in Computer Chemistry* 55:8.

15. Society AC (2015) Cancer Fact and Figures 2015. (American Cancer Society).

16. Ireland P (2015) Pfrixer marks world cancer day with grant to union for international cancer control to address the needs of metastatic breast cancer patients. (Pfizer Ireland).

17. Heldring N, *et al.* (2007) Estrogen receptors: How do they signal and what are their targets. *Physiological Reviews* 87:27.

18. Russo IH & Russo J (1998) Role of hormones in mammary cancer initiation and progression. *Journal of Mammary Gland Biology Neoplasia* 3:13.

19. Alvarez LD, Veleiro AS, & Burton G (2015) Exploring the molecular basis of action of ring D aromatic Steroidal antiestrogens. *Proteins: Structure, Function and Bioinformatics* 83:10.

20. Conzen SD (2008) Minireview: Nuclear receptors and breast cancer. *Molecular Endocrinololy* 22:14.

21. Yang G, Nowsheen S, Aziz K, & Georgakilas AG (2013) Toxicity and adverse effects of Tamoxifen and other anti-estrogen drugs. *Pharmacological Therapy* 139:13.

22. Burendahl S, Danciulescu C, & Nilsson L (2009) Ligand unbinding from the estrogen receptor: A computational study of pathways and ligand specificity. *Proteins: Structure, Function and Bioinformatics* 77:15.

23. Guengerich PF (2005) Human cytochrome P450 enzyme. *Cytochrome P450: Structure, Mechanism, and Biochemistry*, ed Montellano PROd (Plenum, New York), 3 Ed, p 3.

24. Nardo GD & Gilardi G (2013) Human aromatase: Perspectives in biochemistry and biotechnology. *Biotechnology and Applied Biochemistry* 60:10.

25. Banting L & Ahmed S (2009) Aromatase: The enzyme and its inhibition. *Anti-cancer Agents in Medicinal Chemistry* 9:15.

26. Santen RJ, Brodie H, Simpson ER, Siiteri PK, & Brodie A (2009) History of aromatase: Saga of an important biological mediator and therapeutic target. *Endocrine Reviews* 30:33.

27. Park J, Czapla L, & Amaro RE (2013) Molecular simulations of aromatase reveal new insights intro the mechanism of ligand binding. *Journal of Chemical Information and Modeling* 53:10.

28. Plourde PV*, et al.* (1995) ARIMIDEX: A new oral, once-a-day aromatase inhibitor. *Journal of Steroid Biochemistry and Molecular Biology* 53:5.

29. Trunet PF, Bhatnagar AS, Chaudri HA, & Hornberger U (1996) Letrozole (CGS 20267), a new oral aromatase inhibitor for the treatment of advanced breast cancer in postmenopausal patients. *Acta Oncologica* 35:4.

30. Egbuta C, Lo J, & Ghosh D (2014) Mechanism of inhibitor of estrogen biosynthesis by azole fungicides. *Endocrinology* 155:7.

31. Cai J*, et al.* (2015) Computational insights into inhibitory mechanism of azole compound against human aromatase. *Royal Society of Chemistry* 5:10.