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A Novel Development of Cyclic Tetrapeptide as an EGFR Tyrosine Kinase Inhibitor Assayed by ELISA Experiment and Docking Study

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Abstract

The first purpose of this project is to synthesize innovative cyclic peptides and test them as Epidermal Growth Factor Tyrosine Kinase Inhibitor (EGFR-TKI) by Enzyme-Linked Immuno Sorbent Assay (ELISA) experiment. The Cyclic Phe-Phe-Phe-Gly Tetrapeptide (CPTP) exhibited a strong IC_{50} of 55.6 nM while cyclic heptapeptides, μM level. The second purpose is to compare by AutoDock experiment the effectiveness between our most potent CPTP and US Food Drug Administration (FDA) approved erlotinib. The result of a docking study of the CPTP showed a fairy strong inhibition free energy of -7.74 kcal/mol. The two hydrogen bonds were observed between the donor hydrogen of CPTP and the acceptor oxygen of the EGFR residue PHE771, confirming the strong affinity between CPTP and EGFR protein. Cancer reveals the major mortality world-wide. The comprehensive understanding and strategy of cancer is inevitable for the diagnosis, treatment, and prevention of cancer. We hope that the future clinical research will elucidate the fact that our novel CPTP is a resistant free and effective drug for the treatment of Non-Small-Cell (NCS) lung cancer.

Keywords: Cyclic tetrapeptide; Epidermal Growth Factor Tyrosine Kinase Inhibitor (EGFR-TKI), Non-Small-Cell (NCS) lung cancer; Enzyme-Linked Immuno Sorbent Assay (ELISA); Molecular docking

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Background and Introduction

Neoplastic overview

According to the data produced by International Agency for Research on Cancer, there are an estimated 18.1 million cancer cases and 9.6 million cancer deaths in 2018. Lung cancer is the most commonly diagnosed cancer (11.6% of the total cases), and the leading cause of cancer death (18.4% of the total cancer deaths), followed by female breast cancer (11.6%).

Cancer is a major public health problem worldwide and is the second leading cause of death in the United States. In 2020, 1,806,590 new cancer cases and 66,520 cancer deaths are projected to occur in the United States. The cancer mortality rate rose until 1991, then fell every year through 2017, resulting in 29% overall decline. This progress is driven by long-term declines in death rates for the 4 leasing cancers: lung, colorectal, breast and prostate. We believe that chemotherapeutic advancement, one of the major cancer treatment tools; have made a great contribution including computer docking for this progress[1-3].

According to the 2018 global cancer statistics in 185 countries, in both male and female combined, lung cancer is the most commonly diagnosed cancer and the leading cause of cancer death, nearly followed by female breast cancer cases, male prostate cancer cases, and colorectal cancer case [4]. The global burden of cancer, a disease responsible for nearly 10 million deaths per year, is predicted to top 13 million deaths by 2030 in the world [5].

Protein tyrosine kinase

Receptor tyrosine kinases are a large multigene family and the high-affinity cell surface receptors for many polypeptide growth factors, hormones, and cytokines. They have been referred not only to key regulators of normal cellular processes but also to a critical role in the development and progression of many types of cancers. Out of 90 tyrosine kinases assayed 58 are receptor type, and 32 are non-receptor tyrosine kinases. Tyrosine kinase genes can be found on 19 of the 24 human chromosomes. The human tyrosine kinases may be grouped into 20 receptors and 10 non-receptor classes [6].

Protein kinase receptors are note-worthy receptors in the regulation of a variety of cellular processes that catalyze the phosphate group transfer from ATP to a hydroxy group of a serine or threonine. The following different Receptor Tyrosine Kinase (RTK) families have been identified at present [7,8].

1. Epidermal Growth Factor Receptor (EGFR)

2. Insulin Receptor (Insulin R)

3. Platelet-Derived Growth Factor Receptor (PDGFR)

4. Vascular Endothelial Growth Factor Receptor (VEGFR)

5. Fibroblast Growth Factor Receptor (FGFR)

6. Colon Carcinoma Kinase (CCK)

7. Nerve Growth Factor Receptor (NGFR)

8. Hepatocyte Growth Factor Receptor (HGFR)

9. Ephrin Receptor (EPHR)

10. A tyro3 protein tyrosine kinase: from Geek word, annex-elector or uncontrolled (AXL)

11. Tyrosine kinase receptor in endothelial cells (TIE)

12. Receptor related tyrosine kinases (RYK)

13. Discoid in Domain Receptor (DDR)

14. Rearranged during transfection (RET)

15. RPTK expressed in some epithermal cell types (ROS)

16. Leukocyte Tyrosine Kinase (LTK)

17. Receptor orphan muscle specific kinase (ROR)

18. Muscle specific kinase (MUSK)

19. Lemur (LMR)

20. Undetermined

Although there are many types of tyrosine kinase, multi-tyrosine kinase inhibition can be possible and studied by Wang et al., [9]. They used four endometrial carcinoma cell lines, and the activations of tyrosine kinase receptors was analyzed by Western blot, luciferase assay, and immunoprecipitation.

The Epidermal Growth Factor Receptor (EGFR), Human Epidermal growth factor 2 (HER2), and Human Epidermal growth factor 3(HER3) are proto-oncogenes which play important roles in the initiation and progression of human cancers. The homodimerization of EGFR with other Human Epidermal Receptor 1 (HER1) members, and phosphorylation result in the activation of downstream effectors including RAS (Ras protein)-RAF (serine/threoninekinase) -MEK (extracellular signal-regulated kinase) -ERK (extracellular regulated kinase) [10,11]. This EGFR signaling is important cascade deregulating cancer. Once EGFR-bound protein 2 (Grb2) -Son of sevenless (Sos) complex docks to phosphorylated EGFR, the RAS transaction pathway is activated leading to sequential phosphorylation of RAF. And then, MEK and ERK are activated subsequently [12,13]. In other words, this Ras/Raf/MEK/ERK signaling pathway regulates the expression of a vast number of proteins involved in the control of cell proliferation, differentiation, and apoptosis.

Lapatinib is a tyrosine kinase inhibitor which blocks HER1 and HER2 and used for breast cancer patients. It restricts phosphorylation of HER1 and HER2 by competitively inhibiting ATP binding sites of the intracellular kinase region interrupting the downstream signals resulting not only in the induction of the apoptosis but also in the restriction of the development and migration of cancer cells [14]. Xuhong et al., studied mechanism, and safety of tyrosine kinase inhibitors in human epidermal growth receptor 2 positive (HEG 2-positive) breast cancer [14]. They mentioned that the small molecular tyrosine kinase inhibitors such as lapatinib, neratinib, and pyrotinib has taken public attention by the advantages of oral administration, multiple-target therapies, and low cardiotoxicity.

Anti-cancer drugs targeting the above-mentioned Ras/Raf/MEK/ERK Mitogen-Activated Protein Kinase (MAPK) pathway mediates cellular responses in different growth signals. There are three Raf kinases; A-Rad, B-Raf, and C-Ras. However, only B-Raf is often mutated in various cancers. A substitution of a glutamine acid residue to a valine moiety at codon 600 is the most common B-Raf mutation. The MARK pathway is constitutively activated without any growth signals. Novel drugs with inhibiting the activities of Ras and MEK have been developed [15,16]. Taking into consideration of the sophisticated protein tyrosine kinase role, our project was initiated by targeting EGFR.

Docking study

Molecular docking is a computational application to achieve an optimized conformation between a particular protein and a ligand such that the free energy of the overall system is minimized. When used prior to *in vivo* drug screening, the molecular docking can be a powerful computational tool to reduce the labor and cost of the drug development. Users can choose either the generic algorithm or simulated annealing in AutoDock 4.2.6, which is developed by Olson's group [1].

In AudoDock 4.2.6 the overall docking energy of a ligand in the active site is expressed as in equation 1 [1].

$$\Delta G_{(lig)} = \sum_{i,j}\left(\frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^{6}}\right) + W_{hbond}E(t)\sum_{i,j}\left(\frac{C_{ij}}{r_{ij}^{12}} - \frac{D_{ij}}{r_{ij}^{10}}\right) + W_{elec}\sum_{i,j}\frac{q_{iq_j}}{\varepsilon(r_{ij})r_{ij}} + W_{tor}N_{tor} + W_{desoiv}\sum_{i,j}(S_iV_j + S_jV_i)e^{(-r_j^2/2\sigma^2)}$$

$$= \Delta H_{vdw} + \Delta H_{hbond} + \Delta H_{elec} + \Delta S_{tor} + \Delta G_{desolv} \quad \ldots\ldots \text{Equation (1)}$$

The intermolecular potential energies are expressed by the sum of all pairs of ligand atom, i, and protein atom, j, as a function of their distances, r. The potential energies include a Lennard-Johns 12-6 dispersion/repulsion term (ΔH_{vdw}), a directional 12-10 hydrogen bond term (Δ_{hbond}), where E(t), is the directional angle-based weight, a Coulombic electrostatic potential term (ΔH_{elec}) with a distance-dependent dielectric screening (Δ), an entropy of ligand binding term (ΔS_{tor}) that is proportional to the number of sp^3 bonds in the ligand (N_{tor}), and a desolvation term(ΔG_{desolv}) which is a function of the solvent accessible surfaces of ligand (S_i) and protein (S_j).

Cyclic compounds

Cyclic compounds exhibit more rigid structure than non-cyclic compounds so that they can bind to the active site of a specific target without binding to other sites which might produce side effects. Ingangin et al., designed and synthesized pentacyclic peptide, Ser-Arg-Ser-Arg-Tyr, and found that it prevented the

formation of capillary-like tubes of endothelial cells co-cultured with chondrosarcoma cells and trans-endothelial migration of osteosarcoma and chondrosarcoma cells [17].

The sis-oncogene is expressed in some tumor cell lines derived from osteosarcoma and gliomas [18]. Yoshida et al., [19] reported that cyclictetrapeptide, trapoxin, isolated from fungus *Helicoma ambiens*, exhibited a strong effective activity with low toxicity against sis-oncogene [6]. Meditopes are cyclic peptides that bind in a specific pocket in the antigen binding fragment of a therapeutic antibody such as cetuximab [6].

Toyama et al., designed and synthesized bivalent ligands based on a cyclic peptide mimicking the dimerization arm structure of EGFR. Bivalent ligands with polyproline could cause the kinase inhibition triggered by binding of bivalent ligands to two preformed dimeric EGFRs to separate two EGFRs on the cell membrane. They concluded that bivalent EGFR ligands with optimized and rigid linkers could regulate the clusters of the EGFR with higher affinity and suppress kinase activation involving Epidermal Growth Factor (EGF) [20].

Materials and Methods

Solid-phase synthesis of cyclic tetra/penta-peptides originally designed by our laboratory

We made an arrangement with GenScript Co. (860 Centennial Ave. Piscataway, NJ, USA) for the synthesis of cyclic tetra/penta-peptides originally designed by our laboratory. Mass spectrum data received from GenScript are shown in Table 1. In the case of CPTP, the ordinary synthetic procedure partly obtained through the communication with GenScript can be described as follows:

1) Fmoc-Gly-OH amino-acid (1 eq.) was dissolved in 1ml of DMF (N, N-dimethyl formamide). And then, both of 1.4 ml of DIPCI (N,N' diisopropylcarbodiimide)/DMF, and 1.4 ml HODP (1-hydroxybenzotriazole)/DMF, were added to activate Fmoc-Gly-OH amino-acid.

2) 2-chlorotrityl chloridepolystyrene resin (1.2 ml/g loading) was dissolved in 1.5 ml DMS, and filtered after vibrating it 20 min. to swell the resin. This process was repeated three times.

3) The activated Fmoc-Gly-OH amino-acid was delivered into the 2-chlorotrityl chloridepolystyrene resin, and filtered after vibrating it 1.5 hours. This process was repeated three times.

4) 1.5 ml of 20% piperidene/DMS was added to the above 3) filtrate, and filtered after vibrating it 20 min. in order to remove Fmoc residue. The filtrate was washed by 1.5 ml DMF repeatedly until the PH of the solution became neutral. And then, the solution was monitored by Kaiser Test to detect the primary amino residue. It was made sure that the solution was positive.

5) In order to condensate the above 4) residue with the second amino acid, 1 ml of Fmoc-Phe-OH amino acid (1 eq.) was dissolved in 1 ml of DMF. And then, 1.4 ml of DPCI/DMF and 1.4 ml of HOPT/DMF were added to activate the condensed residue.

6) The activated Fmoc-Phe-OH amino acid was delivered to the 2-chlorotrityl chloridepolystyrene resin, and filtered after vibrating it 1.5 hours. This process was repeated three times.

7) 1.5 ml of 20% piperidene/DMS was added to the above 6) filtrate, and filtered after vibrating it 20 min. to remove Fmoc residue. The filtrate was washed by 1.5 ml DMF repeatedly until the PH of the solution becomes neutral. And then, the solution was monitored by Kaiser Test to detect the primary amino residue. It was made sure that the solution was positive in the test.

8) The above steps 5), 6), 7) were repeated to condense the third amino acid residue, Fmoc-Phe-OH, and the fourth amino acid, Fmoc-Gly-OH.

9) Tetra-peptide obtained by the above 8) step was placed in 50 ml Mayer flask to which 8 ml of TFA (trifluoroacetic acid) was added and stirred with magnetic stirrer for three hours in order to separate the tetra-peptide from the resin and remove all of the protecting groups at once.

10) In order to obtain the cyclic peptide from the above 8) chain peptide, the ring was closed by adding 1.4 ml of DIPCI/DMF (DIPCI 1 mmol/ml) and 1.4 ml of HOBT/DMF (HOPT 1mmol/ml).

ELISA experiment

Enzyme-Linked Immuno Sorbent Assay (ELISA) is a solid phase immunoassay first described by Engvall and Perlmann [10]. It is used to detect the presence of a ligand in a liquid sample using antibodies directed against the ligand to be assayed.

The ELISA study has been practiced in various fields. One of the examples with step wise procedure is noted by Engvall et al., As a sensitive and simple method for the quantitative examination of antibodies the tubes coated with antigen are incubated with antiserum followed by an enzyme-labeled preparation of anti-immunoglobulin. The enzyme remaining in the tubes after washing provides the amount of specific antibodies in the serum [21]. ELISA experiment on tyrosine kinase inhibitor is reported by Jiang. A small chemical, ZD6470, selectively inhibits two important pathways; one is Vascular Endothelial Growth Factor (VEGF) receptor-dependent tumor angiogenesis, and the other is the Epidermal Growth Factor (EGF) receptor-dependent cancer cell proliferation. The ability of kinase inhibition of ZD6474 was tested by ELISA method, and the VEGF2 inhibition capacity of ZD6474 was as strong as IC_{50}=40 nM, that of VEGF3, 110 nM, that of RET, 130nM, and EGFR, 500 nM [22].

In our experiments Takara Universal Tyrosine Assay Kit (Takara-bio co,7-4-38 Noji-higashi, Kusatsu-shi, Shiga-Prefecture, Japan) was used. The kit consists of a 96-well microliter plate coated with synthesized peptide (poly Glu-Tyr: 4:1, 20~50 kDa) which can be phosphorylated by kinase standard agent. For the purpose of contrasting a calibration curve, 100,200,341,500,600x10^{-7} units/μL as a converted concentration of tyrosine kinase $p60^{c\text{-}src}$ were prepared. As the standard concentration of tyrosine kinase, 500x10^{-7} units/μL was used. To this kit our CPTP was added to find out the degree of phosphorylation and the phosphorylation process was conducted by adding 10 μL of 40 nM ATP at 37^{0}C for 30 minutes. At the completion of reaction, the supernatant fluid of the plate was removed by decantation, and the plate was washed by Tween TBS four times. And then, 100 ml of a blocking solution was added to the plate which was kept at 37^{0}C for 30 minutes. Then, after the washing by Tween-TBS, 50 μL of the anti-phosphorylation antibody was added to the plate which was kept at 37^{0}C for 30 minutes. The reaction fluid was removed by decantation, and the plate was rinsed by Tween-TBS four times to remove the remaining solution. After the addition of 100 μL of HRP, substrate coloring agent, the plate was kept at 37^{0}C for 15 minutes. As a final step the reaction was terminated by adding 100 μL of 1 N-sulfate solution. The degree of absorbance was read by a microplate reader at 450 nm.

Docking study

Molecular docking study was conducted by using AutoDock 4.2.6 along with AutoDock Tools, both developed by Scripps Research Institute [1]. The computer used was NEC Lavie Windows 10.

The grid box encompassing the binding site, where the ligand was embedded, was created and the grid maps representing the co-crystallized ligand and the receptor protein (pdb code: 1m17) for the actual docking process were pre-calculated using the AutoGrid (included in AutoDock package). The grid box was chosen to be sufficiently large enough to engulf not only the active site but also the significantly pertinent portion of the surrounding surface. The size of grid box was thus designated to be 70x70x70 in Å with a grid spacing of 0.375Å. Since the location of embedded ligand in the complex is known, the cubic grid box was centered on the pivot of the embedded ligand binding site.

After the successful completion of the AutoGrid process, AutoDock was run to calculate the binding free energy between the given inhibitor conformation and the macromolecule which is EGFR, (pdb code: 1m17). Out of the three different algorithms offered by AutoDock, the Genetic Algorithm with Local Search (GALS) was applied for the interaction/binding mode between the macromolecule and the inhibitor. The 100 independent docking runs were performed in order to expand the probability to obtain the better docking conformation. Cluster analysis was performed on the docking run applying a Root Mean Square (RMS) tolerance of 0.5Å. The top ten clusters were ranked from the average lowest energy to the highest. The mode of interaction of the embedded 4-anilinoquinazoline inhibitor, erlotinib, within EGFR kinase, from which RMSD was calculated, was used as a standard docking model.

Results and Discussion

Cyclic Peptide

The p53 tumor suppressor is a powerful transcription factor and play a significant role in the cell cycle regulation, apoptosis, DNA repair, senescence, and angiogenesis [23]. P53 is a significant regulator of cellular physiology in many aspects. It is not only a well-known tumor suppressor, but also a regulator of processes important for maintenance of homeostasis and stress response. Generally speaking, its function is anti-proliferative. When the cell is damaged beyond repair or extremely stressed the p53 protein contributes to apoptosis. Considering the key role in preventing cancer, there is no doubt to state that it is the most frequently mutated gene in human cancer. Unexpectedly, however, a subset of out-of-sense mutations occurring in p53, namely "gain-of-function" causes it to lose its suppressor activity acquires new functionalities that turn the tumor suppressor into an oncoprotein. In other words, it has been demonstrated that increased malignancy of cancers with mutated p53 takes place.

Cyclic peptides have been a valuable research topic in recent years. Wang et al., identified prosaposin as a potent inhibitor of tumor metastasis, which acts via a stimulation of p53, and is the anti-tumorigenic protein throposponrin-1(TSP-1) in bone marrow-derived cells that are recruited to metastatic sites [24]. The transformation from normal to malignant phenotype in human neoplasm is referred to aberrant cell-surface glycosylation. Therefore, targeting glycosylation modules in cancer facilitates the research and development for new molecular probes for bioanalytical and biomedical applications. Taking into this paradigm Rodrquez et al., synthesized lectinomimics based on odorranalectin I; the smallest lectin-like cyclic peptide isolated from the frog *Odorrana grahami* skin, and assessed the ability of these peptides to bind specific carbohydrates on molecular and cellular levels [25].

Sang et al., synthesized monocyclic and bicyclic peptides and reported that those cyclic peptides potently targeted Grb7 (growth receptor bound protein) SH2 (Src homology domain 2). Grb7 promotes both proliferative and migratory cellular pathways through interaction of its SH2 domain with upstream binding partners [26]. However, those are large cyclic peptides and usually not suitable for oral administration. Molecular weights of most of medicines on the market range from 300 to 500. We believe that cyclic tetrapeptide is a smallest cyclic peptide as a drug which can be used for oral administration considering the fact that cyclic tripeptides are exceedingly difficult to synthesize. As far as we understand, due to the difficulty to synthesize and some other problems of cyclic tetrapeptide as a drug research reports on cyclic tetrapeptide have seldom been observed except the report by Peng et al., They reported identification of LY2510924, novel small cyclic peptide that exhibits antitumor activities in solid tumor and breast cancer metastatic models [27]. Cyclic peptides have an advantage as a drug, because they represent geometrically circle and rigid forms which fit favorably in the target pocket of the receptor. Considering those valuable researcher' attempts and our theory, we decided to synthesize cyclic tetrapeptide and cyclic pentapeptide as inhibitor of EGFR tyrosine kinase (Figure 1).

Protein tyrosine kinase

Protein Tyrosine Kinases (PTK) can yield a significant impact on drug resistance through their intervention of anti-apoptotic signal transaction pathways [28]. Some of the notable PTKs are the Epidermal Growth Factor Receptor (EGFR) family which comprises EGFR (ErbB1, HER1), Her2 (ErbB, Neu), Her3 (ErbB3), and Her4 (ErbB4) [29, 30]. ErbB proteins belong to subfamily I of super family Protein Tyrosine Kinases (PTKs). What they have in common include an extracellular ligand-binding domain, a single membrane-spanning region, and a cytoplasmic protein tyrosine kinase domain. The EGF-related peptide growth factor ligands bind the extracellular domain of ErbB receptors leading to the formation of homo- and hetero-dimers. The dimerization eventually triggers the autophosphorylation of specific tyrosine residue in the cytoplasmic domain. Phosphorylated residues serve as docking sites for signaling molecules involved in the regulation of the cascades. Gene expression as downstream effects of

Table 1: IC_{50} of tyrosine kinase inhibition against cyclic tera/penta-peptides obtained by Takarabio Universal Tyrosine ELISA Assay Kit and the theoretical molecular weight and the analyzed molecular weight obtained by Mas spectrum.

Tetra-cyclic and cyclic pentapeptides tested by ELISA assay	IC_{50} of tyrosine kinase inhibition	Theoretical molecular weight	Analyzed molecular weight obtained by Mas spectrum
Phe-Phe-Phe-Gly	55.6 nM	498.6	499.6
Cys-Pro- Gly -Pro-Cys	92.1 μM	455.5	457.2
Pro-Lys-Pro-LysPro	127 μM	547.7	548.6
Tyr-Ala-Val-Ala-Tyr	129 μM	567.6	568

Table 2: Affinity distribution of the docking result of cyclic tetrapeptide, Phe-Phe-Phe-Gly , erlotinib, and chalcone against EGFR tyrosine kinase (pdb code: 1m17).

Compound	Lowest Affinity Distribution	Highest Affinity Distribution	Reference
	Lowest energy (kcal/mol)	Highest energy (kcal/mol)	
1) FFFG cyclic peptide	-7.74	-6.32	current paper
2) Erlotinib	-5.15	-3.31	current paper
3) Erlotinib	-7.6	-	[27]
4) Chalcone	-8.1	-6.9	[27]

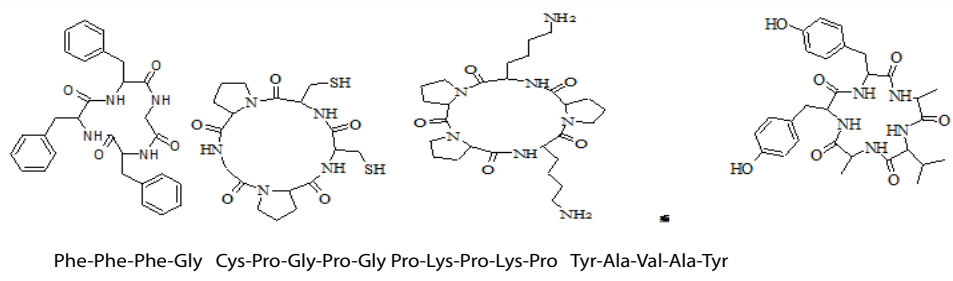

Figure 1: Cyclic tetra/penta-peptides synthesized and tested for the inhibition against EGFR protein tyrosine kinase both by AutoDock assay and ELISA experiment.

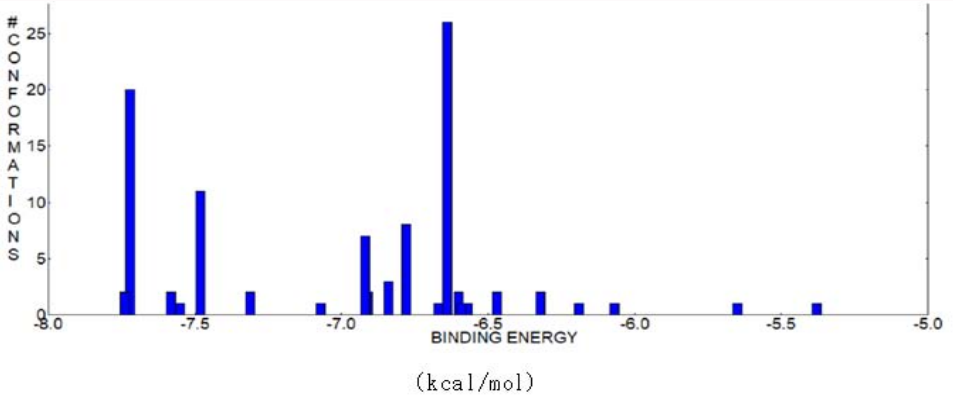

Figure 2: Docking conformation histogram of Phe-Phe-Phe-Phe cyclic peptide against EGFR tyrosine kinase. The lowest binding free energy is -7.74 kcal/mol, and the highest, - 6.32 kcal/mol. The second largest cluster, whose run number is 20 out of the total 100, is formed right next to the lowest energy cluster with run number 2.

the cascade determines the biological response to receptor activation [30]. The existence of Tyrosine Kinase Domain (TKD) is inevitable for the functional activation of the receptor and eventually the induction of the EGFR signaling pathways for the control of cell division and proliferation. The TKD has a bilobate arrangement marked by a C-lobe and an N-lobe, an activation loop. This conformation of molecule renders the accommodation for the binding of the substrate and ATP at the active site, enabling substrate phosphorylation to occur along with the hydration of ATP [31]. Those findings of researchers indicate that there exists an explicit drug target, and from the standpoint of investigation for an effective drug molecule the docking is attractive means as drug development research. Our attempt to explore antineoplastic agent is substantiated referring to the above literature examination and the paradigm of new drug discovery based on particular molecular target.

As many chemotherapeutic treatments become popular, drug resistant problems have been increasing to the un-ignorable level [32,33]. One of the reported resistances to acquired EGFR tyrosine kinase inhibitors is histological transformation from Non-Small Cell Lung Cancer (NSCLC) to Small Cell Lung Cancer (SCLC) [34]. The unexpected findings that a subset of NSCLCs with mutated EGFR return as SCLC when resistance to EGFR tyrosine kinase inhibitors develops. The most common resistance mechanism in this incidence is a Thr790Met mutation in EGFR, which increases the affinity of the receptor for ATP and allows for continued EGFR signaling in the presence of the inhibitor [35]. Another mechanism of resistance is target gene modification which includes EGFR T790M second mutation, alternative pathway activation caused by MET amplification, Hepatocyte Growth Factor (HGF) over-expression, HER2 amplification, PIK3CA mutation, and PTEM loss [34]. In the patient with EGFR T790M second mutation, the DNA sequence of the EGFR gene in his tumor biopsy specimen at relapse revealed the presence of a second point mutation, resulting in threonine-to-methionine amino acid change at position 790 of EGFR leading to gefitinib resistance [36]. MET amplification causes gefitinib resistance by driving ERBB3 (HER3)-dependent activation of P13K, a pathway thought to be specific to EGFR/ERBB receptor [37]. HGF-mediated MET activation is shown to be a novel mechanism of gefitinib resistance in lung adenocarcinoma with EGF activating mutations [38]. HER2 was amplified in 12% of lung adenocarcinomas patients treated with EGFR Tyrosine Kinase Inhibitors (TKI) with

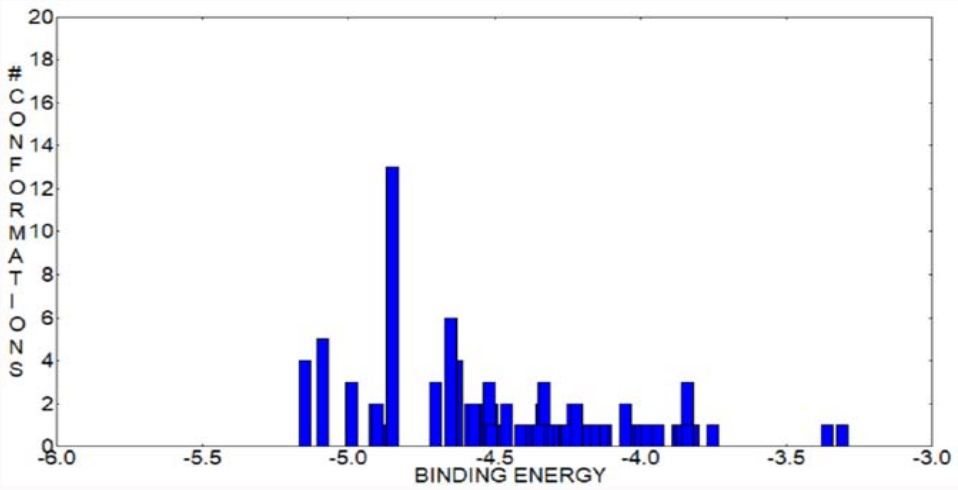

Figure 3: Docking conformation histogram of erlotinib against EGFR tyrosine kinase. The lowest binding free energy is -5.17 kcal/mol, and the highest, -4.31 kcal/mol. The largest cluster, whose run number is 13 out of the total 100 runs, is formed at the sixth cluster those energy medium free energy is -4.56 kcal/mol.

Figure 4: The docking conformation of cyclic peptide Phe-Phe-Phe-Gly against EGFR indicating the hydrogen bond formed between the donor hydrogen of Phe-Phe-Phe-Gly and the acceptor oxygen of PHE771 residue of EFGR. This figure is created by using AutoDockTool [1]. **a)** Hydrogen bond between Phe-Phe-Phe-Gly cyclic peptide and EGFR residue ASP776 with the distance of 2.12 Å. The donor hydrogen is indicated by the ∗yellow sign, **b)** Phe-Phe-Phe-Gly cyclic peptide forming hydrogen bond with residuePHE771 with the distance of 1.87 Å. The donor hydrogen is indicated by the ∗yellow sign and **c)** The conformation table of the docking run NO. 56 out of 100 runs between EGFR and Phe-Phe-Phe-Gly cyclic peptide indicating the binding energy of -7.74 kcal/mol and two hydrogen bonds with EGFR residues PHE771 and ASP776. The donor hydrogen is indicated by the ∗yellow sign.

acquired resistance versus only 1% of the untreated case. Takezawa et al., stated a rationale to assess the status and possibly target HER2 in EGFR-mutant tumors with acquired resistance to EGFR-TKIs [39]. Sequist et al., reported that patients with drug-resistant non-small cell lung cancers showed unexpected genetic changes including EGFR amplification and mutations in the PIK3CA gene, whereas others underwent a pronounced epithelial-to-mesenchymal transition [40]. While additional mechanisms contributing to EGFR inhibitor resistance remain elusive, Sos et al., showed that PTEN loss partially uncouples mutant EGFR from downstream signaling and activates EGFR in EGFR-dependent cells, thereby contributing to erlotinib resistance [41].

Resistance developments to EGFR tyrosine kinase take place ranging from first generation to third generation including gefitinib, afatinib, erlotinib, and osimertinib which are not cyclic-peptide. We believe that one of the approaches to avoid this problem is structure-related strategy. It is highly expected that our approach initiated from a standpoint of small cyclic peptide will reduce the resistance to EGFR tyrosine kinase inhibitors. The flat molecules alternate the geometric feature easily by attacking not only the right pocket but also the false pocket of the target receptor inducing resistances and causing various side effects. On the other hand, cyclic peptides represent the rigid three-dimensional geometry and bind to the right pocket and bind less to the false pocket.

EGFR tyrosine kinase inhibition by the cyclic tetra/penta-peptides in the ELISA experiment

Hepatocyte Growth Factor (HGF) is most invulnerable mitogen against mature hepatic cells. HGF is secreted from the mesenchymal cell and intervene in epidermal cells as multi-function cytokine, and conduces to various physiological influences such as inflammation, angiogenesis, cancer metastasis, immunological modulation against virus, and so on [42]. The explicit activation of the Epidermal Growth Factor Receptor (EGFR) is strongly associated with malignant progression of neoplastic diseases. EGFR belongs to the ErbB (ErbG gene) family of receptor tyrosine kinase, which consists of four members (ErbG1-4). All ErbB family members consist of a common

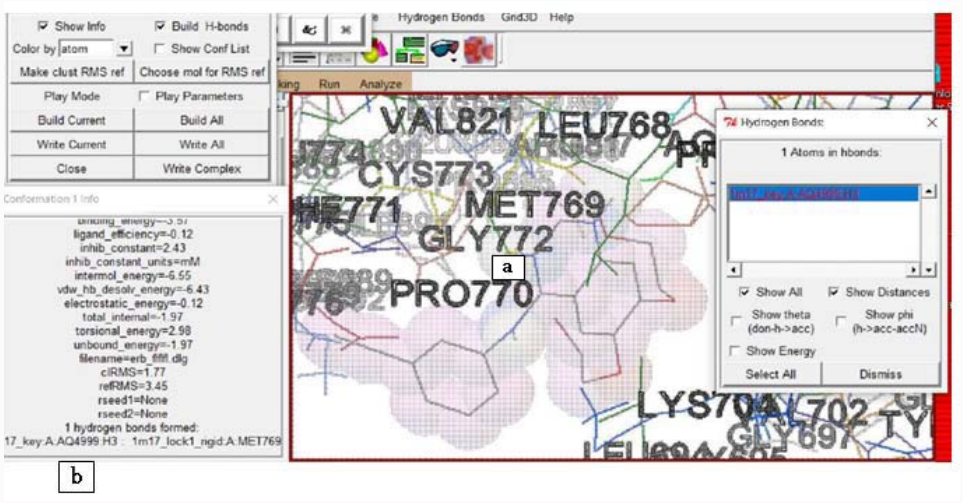

Figure 5: The docking conformation of erlotinib against EGFR indicating the hydrogen bond formed between the donor hydrogen erlotinib and the acceptor oxygen of MET769 residue of EFGR. This figure is created by using AutoDock Tool [1]. **a)** Phe-Phe-Phe-Gly cyclic peptide forming hydrogen bond with residueMET769 (green line) with the distance of 1.97 Å and **b)** The conformation table of the docking run NO. 87 of 100 runs between EGFR and erlotinib indicating the binding energy of -5.15 kcal/mol and one hydrogen bond with EGFR residue MET769. The acceptor nitrogen is indicated by the ∗yellow sign.

structure organization that is constituted of such domains as an extracellular ligand-binding domain, a trans-membrane domain, and an intracellular domain with tyrosine kinase activity. Inhibitors of EGFR have been used in the clinical setting in the treatment of solid tumors. Sun et al., reported that a new synthetic quinonazoline derivatives selectively inhibited EGFR with IC_{50} value of 50 nM [43].

Figure 1 shows cyclic tetra/penta-peptides synthesized and tested for the inhibition of protein tyrosine kinase both by AutoDock and ELISA. Table 1 shows the result of tyrosine kinase inhibitions against cyclic tetra/penta-peptides by the ELISA experiment. CPTP showed the strong nM level of inhibition, while the rest of cyclic pentapeptides, μM levels. This indicates that the receptor pocket of the protein tyrosine kinase is not spacious enough to enclose the cyclic pentapeptides. At the same time, it can be elucidated that the cyclic tetrapeptide fits to the pocket contour of EGFR geometrically in a more suitable way than the cyclic heptapeptides. Taking into consideration of the result of this ELISA experiment we decided to conduct the docking in-silico experiment of cyclic tetrapeptide along with FDA approved erlotinib.

AutoDock study of CPTP against EGFR

Kozakov et al., reported a geometric approach to macromolecule-ligand interactions. Their method is to explore geometrically feasible alignments of ligands and receptors of known structure. They declared that their approach seems well-suited generating conformations for energy refinement programs and interactive computer graphics routines [41]. On the other hand, Goodsell et al., [44] constructed and reported AutoDock which is a suite of C program used to predict the bound conformations of a small, flexible ligand to a macromolecule target of known structure. For the conformation searching with a rapid grid-based method of energy evaluation a simulated annealing technique was applied [45].

AutoDock has been used by a substantial number of researchers for the search of innovative drugs. Ali et al., reported the antitumor drug development study of novel fravin analogs by applying an AutoDock study against protein tyrosine kinase $pp60^{c\text{-}src}$ along with an inhibitory experiment against tumor cell lines represented by IC_{50} (μM), where a good correlation between their IC_{50} and AutoDock binding free energy was validated [46,47]. Reddy et al., reported docking analysis of chalcone derivatives including 4-anilinoquinazoine inhibitor, erlotinib, into the ligand binding domain of EGFRtyrosine kinase (PDB code: 1m17) in search of anticancer agents. Their docking free energy obtained for anilinoquinazoline, erlotinib, indigenous inhibitor, was -7.6 kcal/mol, and those of chalcone derivatives were ranged from -6.9 to -8.1 kcal/mol which are an impressive result [48]. Our docking result of the lowest free docking energy obtained for anilinoquinazoline, erlotinib, was -5.15 kcal/mol as shown in Table 2, and the one for cyclic tetrapeptide against EGFR was -7.74 kcal/mol. Both results indicate that chalcone derivatives and CPTP are potentially effective antineoplastic agents to treat NSC lung cancer. Figure 2 shows the clustering histogram of the docking result of CPTP against the EGFR kinase. While Figure 3, that of 4-aniquinazo inhibitor, erlotinib.

As far as the evaluation of the docking result is concerned, the eminent criteria to apply for the selection of valuable drug-like compounds are the lowest free energy, the existence of cluster in the lower free energy region and the existence of H-bond. One of the reliabilities of the AutoDock result is the existence of the similarity of its final docking conformations in the lower binding free region. The method AutoDock applies for this purpose is to measure the reliability of a result by comparing the RMSD of the lowest energy conformations and to group them into families of similar conformations or "cluster." By default, AutoDock clusters the docking results at 0.5Å RMSD [49]. Clustering is a valuable tool in computational biology. Clustering is said to occur where long range electrostatic and desolvation forces steer the protein to a lower free binding energy attractor. Therefore, recurrence of structural motifs is considered to be one of the most suitable determinant of a good conformation [50]. The histogram of our docking result represents 20 clusters among 100 runs, and 20 docking conformations is converged in the second lowest cluster with the lowest binding energy of -7.72 kcal/mol. This indicates that the lowest free energy we obtained is reliable. The histogram of erlotinib is rather scattered in a wide range, and the highest cluster is not created in the lower energy region, but

in the seventh lower energy region. In addition, it can be postulated that the lower docking free energy obtained for CPTP compared to erlotinib elucidates the fact that CPTP is superior to erlotinib as a drug to treat NSC lung cancer.

The closer examination of docking study shows that two hydrogen bonds are formed between CPTP and two EGFR residues, ASP776 PHE771 as shown in Figure 4. The hydrogen bond between the donor hydrogen of CPTP and acceptor oxygen of ASP776 residue was constituted with the distance of 2.12Å. Another hydrogen bond between the donor hydrogen of CPTP and acceptor oxygen of PHE771 residue, with the distance of 1.87Å. The examination of the docking result of erlotinib revealed that only one hydrogen bond is formed between erlotinib donor hydrogen and MET769 residue of EGFR tyrosine kinase (Figure 5). This result substantiates the paradigm that CPTP could be superior to erlotinib for the treatment of NCS lung cancer.

Erlotinib used for unresectable non-small cell lung cancer improves overall survival by 19%, and improved Progression-Free Survival (PFS) by 29%, when compared to a conventional chemotherapy [51,52]. FDA approved erlotinib for the treatment of locally advanced or metastatic non-small cell lung cancer that has failed in at least one prior chemotherapy regimen [53].

Conclusion

Cancer is the leading cause of death worldwide, and the comprehensive understanding of its mechanism of action, various treatment strategies and stringent combats against its elevated morlarity, and mortality is essential including strategy to develop safe and effective antineoplastic agents. Our novel CTPT exhibited strong IC_{50} of 55.6 nM. The molecular docking study of CTPT revealed the tight affinity against EGFR protein representing low free energy of -9.60 kcal/mol. All of the currently available EGFR inhibitors including gefitinib, afatinib, erlotinib, and osimertinib are non-cyclic in their chemical structure, and develop resistance to EGFR inhibitors. Various therapeutic strategies to overcome those resistances of currently available EGFR inhibitors and to thwart its emergence ended up with failure. It is our sincere hope that our novel tetracyclic peptide will be safe and effective agent without producing resistance for the treatment of non-small-cell lung cancer.

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