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CASE STUDY REPORT

Designing PROTACS for HIV-TAT using deep learning and molecular modeling methods

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Date	Author	Comments	Version

1. Abstract

Human immunodeficiency virus infection and acquired immunodeficiency syndrome (HIV/AIDS) are a spectrum of conditions caused by infection with the human immunodeficiency virus (HIV), a retrovirus. During this disease, the immune system gradually breaks down, allowing life-threatening opportunistic infections to thrive. In fact, those, such as cryptococcal and tubercular meningitis, pneumocystis, and fungal and viral infections, are cited as major causes of death, when AIDS is unchecked and uncontrolled. In sum, opportunistic infections (OIs) remain an ongoing threat to the immune system, affecting the patient's health during AIDS, and leading to death.

Once diagnosed as HIV-positive, the patient immediately receives antiretroviral therapy (ART). Even at ART, the immune system loses its ability to clear pathogens from the body and this results in opportunistic infections being fatal. At the centre of this problem, in part, lies macrophages. As part of the immune response, macrophages capture and digest foreign bodies (i.g. phagocytosis). However, in HIV/AIDS, macrophages get infected and over time, they become a reservoir for viral particles. As a result, the phagocytosis capacity of macrophages is greatly diminished. Furthermore, long-lived HIV reservoirs in macrophages persist at ART and continue to contribute to disease pathology. Also, considering the incidence of undiagnosed infections shortly after ART, it is apparent that current HIV treatments fall short in the fight against OIs in HIV/AIDS. To overcome these drawbacks of ART, we propose to develop first-generation therapeutics that help the immune system cope with these infections.

For this, we postulate that the Tat protein in macrophages could be targeted. Tat (transactivator of transcription) exerts many adverse effects during AIDS; however, the most pivotal one is the inhibition of phagocytosis in macrophages. As a result, these white cells lose their ability to destroy pathogens and the immune system collapses. In this regard, we propose that any therapeutic agent that interferes with this role of macrophages can restore the phagocytosis capacity of macrophages and thus reactivate the immune system against opportunistic infections. In this way, the viral load in the body, and thus, the mortality rate in HIV/AIDS will be reduced.

To overcome this challenge, we propose to develop PROTAC for Tat as a new therapeutic approach. PROTACs (proteolysis-targeting chimaeras) are molecules that bind E3 ubiquitin ligases to target proteins, triggering protein degradation by the ubiquitin-proteasome system (UPS) [5]. Designed PROTAC for Tat diverges from any FDA-approved drug or other anti-HIV agents in that it will be the sole therapeutic to accomplish the following: to help the immune system fight the infectious disease during HIV/AIDS.

Proje Özeti

İnsan bağışıklık yetmezlik virüsü (HIV) enfeksiyonu ve edinilmiş bağışıklık yetmezliği sendromu (AIDS), retrovirüs olan insan bağışıklık yetmezlik virüsü (HIV) enfeksiyonu sonucu ortaya çıkan bir dizi durumu ifade eder. Bu hastalık sürecinde, bağışıklık sistemi giderek zayıflar ve yaşamı tehdit eden fırsatçı enfeksiyonlara izin verir. Özellikle, kriptokokal ve tüberküler menenjit, pnömokistis, mantar ve viral enfeksiyonlar gibi enfeksiyonlar, AIDS kontrolsüz ve denetimsiz olduğunda başlıca ölüm nedenleri olarak belirtilir. Sonuç olarak, fırsatçı enfeksiyonlar (FEn), bağışıklık sistemi üzerinde sürekli bir tehdit oluşturur, AIDS sırasında hastanın sağlığını etkiler ve ölüme neden olur.

HIV-pozitif olarak teşhis edildiğinde, hasta hemen antiretroviral tedavi (ART) alır. ART kullanılsa bile, bağışıklık sistemi vücuttaki patojenleri temizleme yeteneğini kaybeder ve bu da fırsatçı enfeksiyonların ölümcül olmasına neden olur. Bu sorunun bir kısmı, makrofajların merkezindedir. Bağışıklık tepkisinin bir parçası olarak, makrofajlar yabancı cisimleri yakalar ve sindirir (yutma işlemi). Ancak, HIV/AIDS durumunda, makrofajlar enfekte olur ve zamanla viral partiküller için bir rezervuar haline gelir. Sonuç olarak, makrofajların yutma kapasitesi büyük ölçüde azalır. Dahası, makrofajlardaki uzun ömürlü HIV rezervuarları ART sırasında persiste olur ve hastalık patolojisine katkıda bulunmaya devam eder. Ayrıca, ART'dan hemen sonra teşhis edilmemiş enfeksiyonların insidansını düşünerek, mevcut HIV tedavilerinin HIV/AIDS'teki FEn'lerle mücadelede yetersiz kaldığı açıktır. ART'ın bu dezavantajlarını aşmak için, immün sistemin bu enfeksiyonlarla başa çıkmasına yardımcı olacak birinci nesil terapötikler geliştirmeyi öneriyoruz.

Bu doğrultuda, makrofajlardaki Tat proteini hedeflenebilir. Tat (transkripsiyonun transaktivatörü), AIDS sırasında birçok olumsuz etkiye sahiptir; ancak en önemlisi, makrofajlardaki fagositozun inhibisyonudur. Sonuç olarak, bu beyaz hücreler patojenleri yok etme yeteneklerini kaybeder ve bağışıklık sistemi çöker. Bu bağlamda, makrofajların bu rolüne müdahale eden herhangi bir terapötik ajanın, makrofajların fagositoz kapasitesini geri kazandırabileceğini ve böylece bağışıklık sisteminin fırsatçı enfeksiyonlara karşı yeniden etkinleştirilebileceğini öneriyoruz. Bu şekilde, vücuttaki viral yük ve dolayısıyla HIV/AIDS'teki mortalite oranı azaltılacaktır.

Bu zorluğun üstesinden gelmek için, Tat için PROTAC'ın yeni bir terapötik yaklaşım olarak geliştirilmesini öneriyoruz. PROTAC'lar (proteoliz hedefli kimeralar), hedef proteine bağlanarak protein parçalanmasını E3 ubikitin ligazları ile tetikleyen moleküllerdir, bu da ubikitin-proteazom sistemi (UPS) aracılığıyla proteinin parçalanmasını sağlar. Tasarlanmış Tat için PROTAC, herhangi bir FDA onaylı ilaç veya diğer HIV karşıtı ajanlardan farklı olarak, aşağıdakileri gerçekleştirmek için tek terapötik olacaktır: bağışıklık sisteminin HIV/AIDS sırasında enfeksiyonla mücadele etmesine yardımcı olmak.

2. Problem Identification

HIV (Human Immunodeficiency Virus) is a virus that attacks the immune system and is one of the biggest public health problems in the world. Human immunodeficiency virus type 1 (HIV-1) is responsible for acquired immunodeficiency syndrome (AIDS). It is a sexually transmitted disease, but an infected woman can transmit her infection to her unborn child during pregnancy, childbirth, or breastfeeding. Sharing injection equipment can also result in non-sexual transmission. As the disease progresses, HIV infects the vital cells of the immune system - T helper cells (CD4+T cells, T cells that contain the CD4 marker), macrophages, and dendritic cells (which present antigens to T cells). The phagocytosis capacity of macrophages (*i.g* the ability to remove pathogens) is also impaired in HIV/AIDS. **As a result, the number of CD4+T cells decreases, cell-mediated immunity is lost, and the body becomes susceptible to infections, leading to AIDS** [1]**.**

In 1981, the world's first HIV/AIDS case was reported in the United States. Between 1981 and 2000, there were approximately 36.1 million people infected with HIV. During this period, there were 21.8 million deaths. According to UnAIDS (United Nations Programme on HIV/AIDS), there will be approximately 38.4 million HIV-positive persons worldwide in 2021. This includes 1.7 million children and 36.7 million adults. By 2021, an estimated 1.5 million people worldwide will be infected with HIV; 1.3 million of the 1.5 million new HIV infections occurred in adults, while 160,000 occurred in children [2]. For this reason, significant amounts of money are being allocated to HIV research; for example, funding agencies around the world have awarded $24.2 billion to researchers conducting research on AIDS (as of June 2022) [8]. **These numbers make it clear that HIV remains a major health problem.**

Since the 1980s, advances in antiretroviral therapy (ART) have been very promising and even led to AIDS becoming a manageable disease. It is worth noting that with HIV, a weakened immune system increases vulnerability to opportunistic infections (such as cryptococcal and tubercular meningitis, pneumocystis, and fungal and viral infections), as well as other conditions such as cancer. This phenomenon predisposes to different coinfections or superinfections and increases their severity [3]. This means that immunosuppression resulting from HIV places a patient at risk for infection from organisms that would normally be cleared by the immune system. It is worth noting that even in the ART era, HIV-related opportunistic infections are still major complications, which may be the result of late diagnosis and low levels of CD4+ T-cell counts [4]. **In short, patients still need to protect themselves from opportunistic infections and to date, these infections accompanying HIV/AIDS are the main reason for death during this disease.**

One of the key reasons leading up to opportunistic infections is the ability of HIV to infect macrophages. By definition, macrophages, which are derived from circulating monocytes, play an important role in the innate and adaptive immune response. As an arsenal against HIV/AIDS, they play many roles. One particular role is that once activated, they eat and remove cellular debris and pathogens through phagocytosis. However, various viruses, analogously to some microorganisms, especially intracellular ones, can adversely affect phagocytosis in these cells. Over time, they get infected and eventually become viral reservoirs, storing HIV-1

particles in internal compartments. This way, the virus particles cover themselves and thus, ART is fully neutralised. **In other words, ART is ineffective against long-lived HIV reservoirs in macrophages. Consequently, macrophages become targets for AIDS-relevant pathogens, thereby fueling the establishment of OI associated with the progression of AIDS** [5]. Today, OIs, such as cryptococcal and tubercular meningitis, pneumocystis, and fungal and viral infections, are counted as the major reason of death in HIV/AIDS.

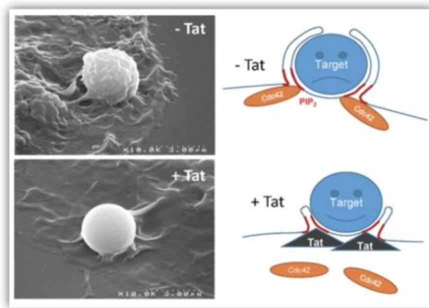

Figure 1. Monocyte-derived human primary macrophages treated with Tat, before adding latex beads. In the absence of Tat protein, CDC42 is triggers actin polymerization and pseudoknot formation. Thus, beads are entirely engulfed by macrophages (upper panel). When present, Tat impairs this mechanism; actin polymerisation is inhibited, and beads are no longer engulfed (lower panel).

Tat protein (transactivator of transcription) is proposed to be the right target to design therapeutics which restore phagocytosis and ameliorate immunity. Encoded by HIV, Tat is a pivotal protein in the life cycle of the virus. This is an intrinsically disordered protein synthesized in the early phase of viral infection [6]. Then, it is secreted by T-infected cells circulating in the bloodstream. Subsequently, it is taken up into uninfected cells, where it exerts many effects, resulting in the collapse of the immune system. **For instance, Tat inhibits the mannose and Fc-gamma receptor-mediated phagocytosis in macrophages.** In uninfected cells, phagocytosis is triggered when CDC42 (Cell division control protein 42) is recruited to the cell membrane by PI4,5P2 (phosphoinositol-4,5-biphosphate). This is followed by actin polymerization and the formation of a phagocytic cup. Once captured by macrophages, pathogens are encapsulated in this cup, taken up into cells and directed to the proteasome where they are degraded. Through this mechanism, macrophages can remove pathogens. In infected cells, Tat competes with CDC42 for PI4,5P2 in macrophages; thus, actin polymerization is largely inhibited, and phagocytosis is largely shut down (**Figure 1**) (7). **Even with successful ART, Tat is produced by infected cells (8); thus, it is a steady threat to the immune system, paving the way to OIs and resulting in the death of the patient.**

To date, the idea of restoring phagocytosis in macrophages has not been explored. The key reason hampering this objective is that Tat is a disordered protein and therefore, there are no secondary structures or binding sites on it. As a result, it was not possible to develop a compound that inhibits any process involving Tat. One exception is the transcription of viral RNA. Here, the Tat protein binds to an RNA stem-loop structure and increases the production of full-length viral RNA. However, so-called anti-Tat agents bind to the Tat/TAR (The HIV trans-activation response) complex, which presents a binding site, instead of Tat [9]. Thus, targeting Tat has always been a major challenge and ultimately; it guided the direction that antiviral therapy is headed. That being so, reverse-transcriptase and protease shined out as the

pertinent targets for antiviral therapy, whereas Tat dropped off the radar, despite its significance.

To tackle this problem, we propose the development of proteolysis-targeted chimaeras (PROTACs) for Tat. PROTACs is a strategy that utilizes the ubiquitin-protease system to target a protein of interest (POI) and induce its degradation (**Figure 2**). In doing this, PROTACs recruit the protein of interest (POI) and an E3 ubiquitin ligase (E3), whereupon a tertiary complex (POI: PROTAC: E3 ligase) is formed. In this ubiquitination machinery, E3 ligase targets POI and covalently attaches the ubiquitin to it. Once polyubiquitinated, POI is recognized and degraded by the 26S proteasome, which is part of the ubiquitin-proteasome system (UPS) in eukaryotic cells [10]. It is also worth noting that PROTAC can catalyze multiple rounds of protein degradation. This catalytic ability of PROTACs is particularly significant in this context as Tat is steadily produced by infected cells.

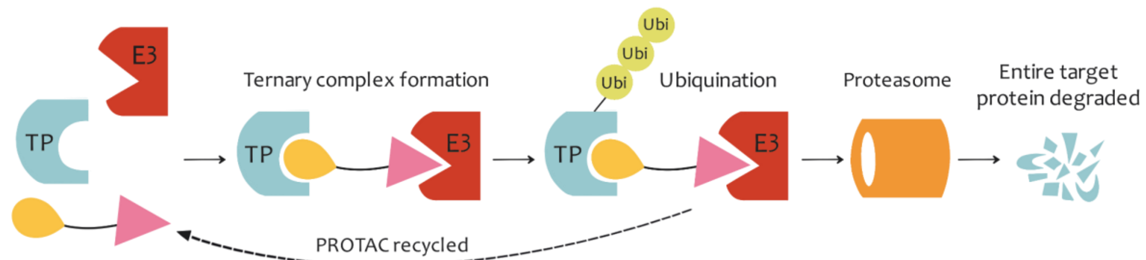

Figure 2. The working principle of PROTACs

The development of PROTAC for Tat will have positive socioeconomic and psychosocial effects on patients. Driven by this rationale, this proposal which aims to increase the ability of macrophages to cope with infections aligns perfectly with the progress of HIV treatment. This is because these infections remain an important problem in patients who are unaware of their HIV serologic status, in those not receiving ART, and even in those who are receiving ART due to poor adherence, failure and cross-resistance. UnAIDS indicates that just in 2017, 940,000 people died from HIV/AIDS-related illnesses [11]. These deaths occur among people who interrupted treatment or were diagnosed with HIV and OIs when they were already too ill to benefit from ART. **Thus, we propose that the development of a PROTAC for Tat will enable the body to cope with opportunistic infections, increase the patient's quality of life and reduce the risk of death from HIV/AIDS. Collectively, we propose that PROTAC for Tat has high therapeutic value to aid the immune system fight opportunistic infection.**

The key to developing PROTAC lies in modeling the tertiary complex, which consists of the target protein to be degraded (TP), the PROTAC molecule selective for TP (e.g. Tat protein in our case), and the E3 ligase enzyme responsible for the ubiquitination of the target protein. This complex brings these two proteins in proximity and appropriate conformation, allowing the E3 ligase to trigger the ubiquitination of the Tat protein. In this understanding, PROTAC itself is comprised of three components, as well: one ligand specific to the E3 ligase, one ligand specific to Tat protein, and a linker bridging these two ligands. Thalidomide or lenalidomide (a derivative of thalidomide) are well-documented ligands for E3 ligases (**Figure 3**). Regarding

that for Tat protein, our initial consideration was TAR RNA, as it is the primary interaction partner of Tat protein during HIV pathogenesis; as such, TAR RNA has been used as a ligand in many applications involving Tat recognition. However, due to the relative complexity of modeling such a large nucleic acid, we decided to revise our strategy and consider Didehydro-cortistatin A (dCA), a potent Tat inhibitor. Being an analog of the natural product cortistatin A (CA), dCA is observed to bind to the 48-59 region, also known as the basic patch of the Tat protein, with a reported EC of 1nM. As for the linker between these two ligands, numerous moieties whose lengths vary between 8 and 16 atoms are designed according to the relevant literature. **With the structure of PROTAC established, the primary challenge has been the construction of TP - PROTAC - E3 ligase tertiary complexes, prior to MD simulations.**

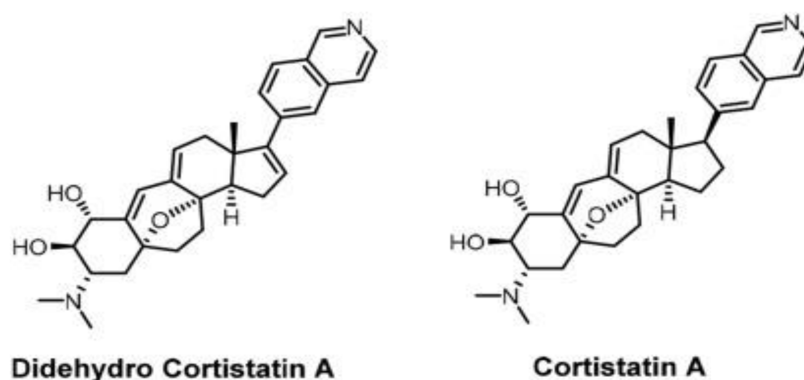

Figure 3. The structures of Didehydro-cortistatin A and Cortistatin A.

3. First Suggestion

To construct the tertiary complexes, our first choice was using scripts or AI-based tools. In this respect, the methodology reported by Zhang et al. was initially considered [12]. This research team introduced an innovative deep generative model for the systematic design of PROTACs, particularly in resource-constrained environments. Their approach involved utilizing deep reinforcement learning to guide the generation of PROTACs with optimized pharmacokinetic properties. By applying this method to target the bromodomain-containing protein 4, the researchers evaluated 5000 compounds and eventually proposed four PROTAC molecules in only 45 days. However, our attempts to run the Python script were unsuccessful. Next, we directed our focus towards the PRosettaC tool, developed at the Weizmann Institute of Science [13]. This server is specifically engineered to yield tertiary complexes from the PDB (protein database) files of target protein-ligand complexes and SMILES code of linkers. Notably, it offers a very user-friendly interface, simplifying the modeling process. Despite our endeavors, we were once again unable to generate the tertiary PROTAC models.

The tentative project plan is as follows:

Timeline	Tasks and Milestones
1-2	Prediction of candidate linkers using artificial intelligence
3-4	Modelling of the ternary complex of E3 Ligase, the predicted PROTACs, and HIV TAT.
5-6	Simulations of the ternary complex of E3 Ligase, the predicted PROTACs, and HIV TAT.

4. Solution Stage – I

Due to numerous technical challenges encountered, the approach was revised to generate PROTAC tertiary complexes via protein-protein docking. To this respect, the Tat protein (PDB: 1K5K, model 1) was complexed with E3 ligase (PDB: 1K5K) using protein-protein docking servers, such as ZDOCK [14] and HDOCK [15]. The PROTAC was then constructed by incorporating the linker and Didehydro-cortistatin A onto the crystal structure of Lenalidomide, using PyMol tool [16]. Subsequently, the PROTAC was docked onto the first ten models of the protein complex obtained from each server. However, it was observed that Didehydro-cortistatin A and Lenalidomide didn't bind to their respective binding sites in these models. Consequently, the anticipated tertiary complexes involving Tat protein, PROTAC, and E3 ligase were not successfully obtained through this approach.

5. Results and Achievements

Ultimately, we decided to "manually" create tertiary complexes after technical problems we have faced. This approach involved obtaining complexes of Didehydro-cortistatin A/Tat and Lenalidomide/E3 Ligase, and then fusing them while constructing the linker in between. The most crucial aspect of this approach is to identify how the linker will be grafted on organic compounds to afford the intact PROTAC. In the binding pose of Lenalidomide to E3 ligases, it is noted that the dicarboximide group is embedded in the binding site, whereas the isoindolin-1-one group faces bulk water. Therefore, in PROTAC design, the linker is attached to the amino or hydroxyl functional group at the 4th position on isoindolin-1-one [17]. However, the binding mode of Didehydro-cortistatin A to Tat is not as straightforward as that of Lenalidomide in that the entire Didehydro-cortistatin A molecule seem to interact with the 49-57 region on Tat. Nevertheless, during literature research, we see that in commercially available biotin-dCa conjugate, biotin is tethered to dCa via hydroxyl group in allylic position to the double bond on cyclohexane. Therefore, we decided to use the same approach to tether the linker onto dCa (**Figure 4**).

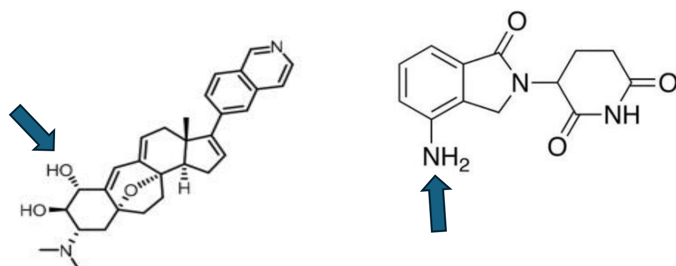

Figure 4. The structures of Didehydro-cortistatin A and Lenalidomide where the attachment sites of linkers are marked.

Subsequently, our goal was to first attach the linker to Lenalidomide and then fuse it with Didehydro-cortistatin A complex, using the PyMol tool. However, the complication we experienced in this approach is that the hydroxyl group, which is the attachment site on dCa, appears embedded within the Tat protein and therefore extends away from the E3 ligase. For this reason, we decided to attach a short linker with four carbons to this functional group to mimic full linker. This way, this hydroxyl group is forced to orient towards bulk solution, allowing conjugation to Lenalidomide. After a 12-carbon linker is grafted onto Lenalidomide, we finally obtained Tat/dCa and E3 Ligase/Lenalidomide complexes, which are then fused to yield Tat – PROTAC – E3 ligase tertiary complex. By altering the linkers, we formed a library of 15 PROTACs. Of note, these linkers we choose for this study are dicarboxylic acids, hydrocarbons, and ethylene glycol as they are preferred in the design of PROTACs (**Figure 5**).

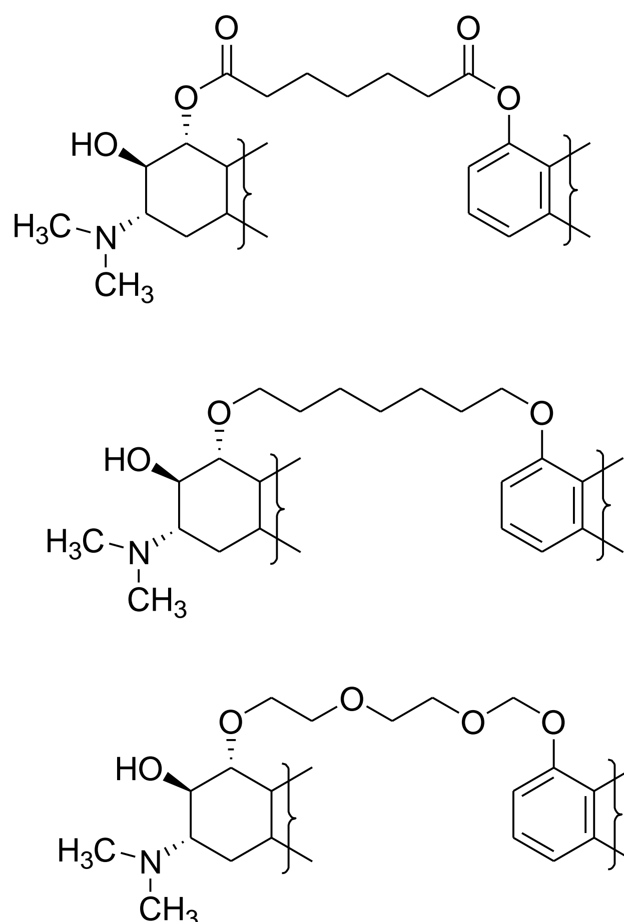

Figure 5. The structures of PROTACs where the linkers are made up of dicarboxylic acid (top), hydrocarbon chain (middle) and ethylene glycol chain (bottom). In these PROTAC designs, dCa and lenalidomide are on the left and right hand side, respectively and partly shown in this figure for clarity.

Despite the technical issues we encountered, PROTAC structures were successfully constructed within the scope of this project. As mentioned in the previous section, 15 PROTAC structures were created by changing the linker molecule, and simulation files for these structures were prepared (**Figure 6**). Subsequently, 1000 ns MD simulations of these structures were carried out, and 5 of these simulations were completed and are currently analysed.

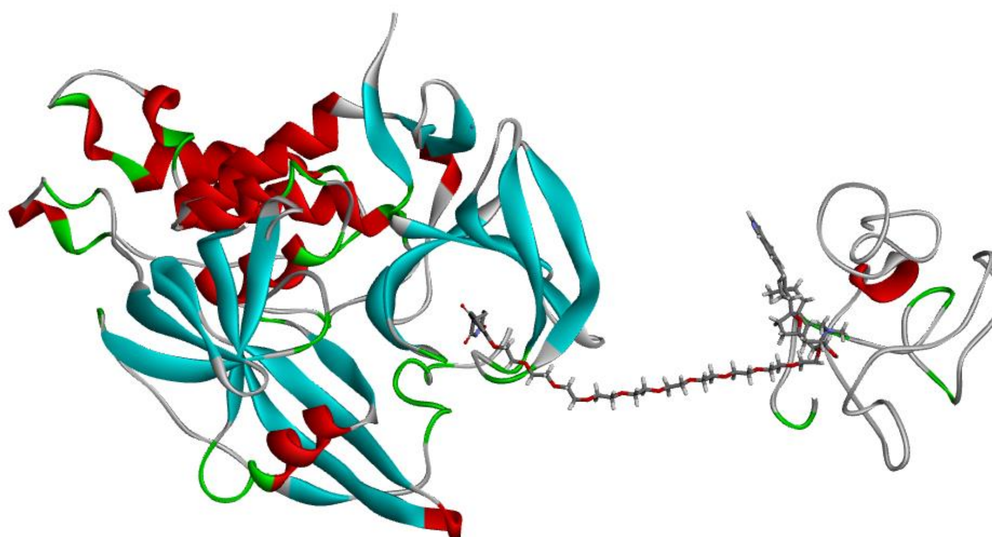

Figure 6. The structure of one tertiary complex that involves E3 ligase, PROTAC, Tat protein (from left to right)

MD simulations were performed with Desmond simulation package. Simulation systems contain approximately 90K atoms. Simulations were performed at 298K and 1 atm conditions. SPC water model and OPLS2005 force-field are used in the simulations. Atomic coordinates were recorded with 20ps intervals. 9 Å cut-off distance is applied. Nosé–Hoover thermostat and the Martyna–Tobias–Klein barostat are used for temperature and pressure control. The simulation time step was 2 fs with the r-RESPA integration method utilized. The overall flow chart is shown in the **Figure 7**.

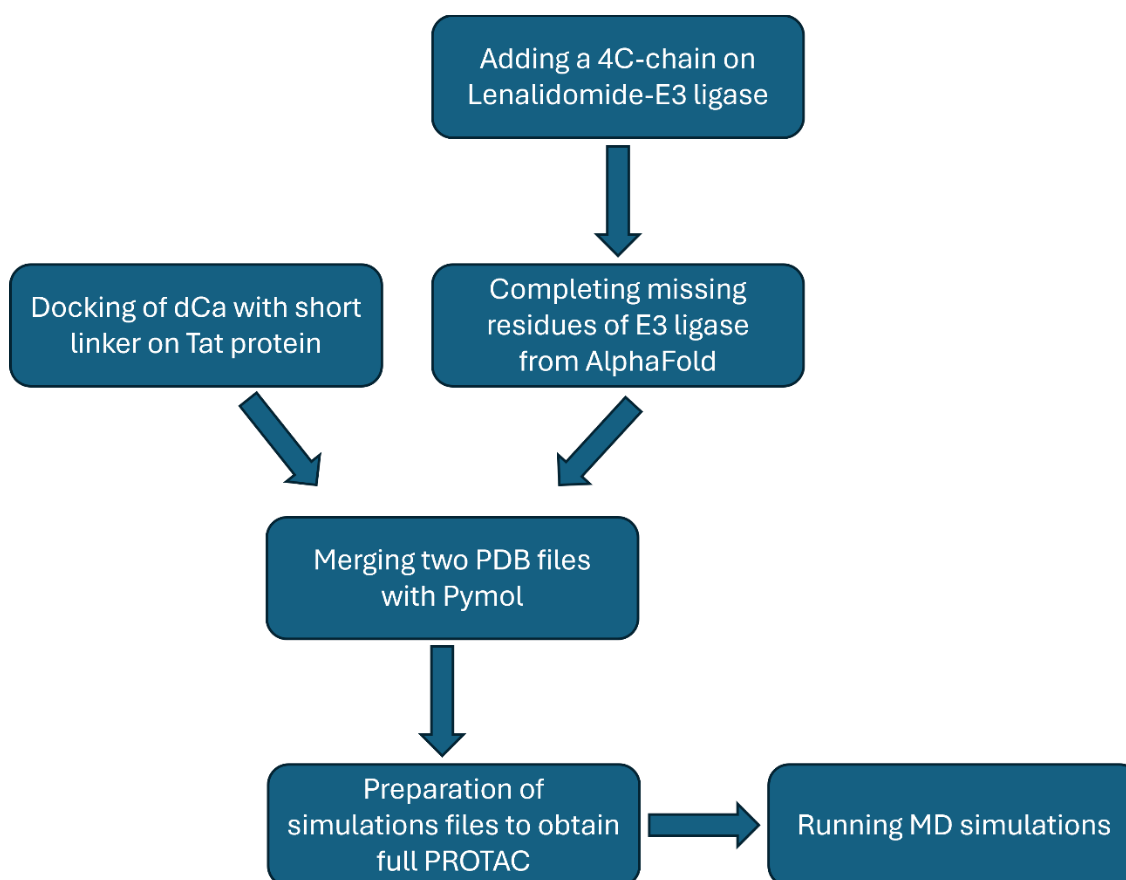

Figure 7. Flow chart of tertiary complexes of PROTACs and MD simulations.

6. Business Benefits

The PROTAC approach, which emerged in the early 2000s, has evolved to a point where it is widely used in the degradation of many proteins or enzymes that are key to diseases, ranging from cancer to neurological diseases. As a result of the dizzying developments in this field, leading pharmaceutical companies around the world have begun to develop PROTAC-based drugs, some of which reached Phase I/Phase II trials (**Table 2**) [18]. Therefore, the scope of this EuroCC project aligns with the current state of PROTAC technology. Through this project, PeptiSyn has accumulated knowledge in designing PROTACs through in silico methods, which is of great importance in pharmaceutical and medicinal chemistry. Upon the

completion of this project, PeptiSyn has now the potential to provide consultancy services to pharmaceutical companies and offer the design of such molecules as a service when needed.

Target	PROTAC name	Types of clinical trials	Patient population	ClinicalTrials. gov number	Sponsor
AR	ARV-110	Phase 1/2	mCRPC	NCT03888612	Arvinas
AR	CC-94676	Phase 1	mCRPC	NCT04428788	Celgene/BMS
ER	ARV-471 alone or with palbociclib	Phase 1/2	$ER^{+}/HER2^{-}$ advanced or metastatic breast cancer	NCT04072952	Arvinas
BTK	NX-2127	Phase 1	Relapse/refractory B-cell malignancies	NCT04830137	Nurix Therapeutics
BCL-xL	DT2216	Phase 1	Relapse/refractory solid and hematologic malignancies	NCT04886622	Dialectic Therapeutics

Table 2. Summary of ongoing clinical trials of PROTAC degraders [18].

7. Challenges

As previously noted, the main challenge in our project has been the generation of PROTAC models. Numerous studies in the literature have addressed this issue, followed by the selection of appropriate linker molecules, and the determination of the pharmacokinetic properties of PROTACs. These studies have commonly utilized servers such as Prosetta C or scripts written in the Python environment. In the first month of the project, an exhaustive literature review was conducted, and a comprehensive list of all utilized codes and servers was compiled. However, despite our efforts in subsequent months, we encountered difficulties in executing these codes and accessing the servers. For instance, we were unable to install the necessary dependencies for the method initially addressed, which was published in the journal Nature. Subsequently, despite dedicating a month to the effort, the Prosetta C server, which we attempted to utilize, failed to generate any PROTAC ternary complexes, despite its seemingly straightforward usability. In attempting to resolve the technical issues encountered, we made multiple attempts to contact the creators of the server but received no response from them. Consequently, we were compelled to manually generate these complexes. This situation constituted the most significant technical challenge within the scope of the conducted project.

8. HPC Usage Status

MD Simulations were performed on akya machines. In each simulation one node, 40 CPU cores and 4GPUs, is used. Simulations of several molecular systems were performed in parallel.

9. Literature Cited

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