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In *Silico* Study of Anticancer and Antimicrobial Peptides Derived from Cycloviolacin O2 (CyO2)

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Abstract: Cancer is considered a global threat to public health as one of the leading causes of premature deaths in most countries worldwide. Chemotherapy in cancer has reached a therapeutic plateau caused by high cost, low specificity, long development time, and severe side effects. Currently, peptide-based targeted therapeutics are being extensively studied to be utilized for cancer treatment due to their small size, high specificity, and selectivity. In this study, we utilized several *in silico* methods to design more potent dual anticancer and antimicrobial activities using Cycloviolacin O2 (CyO2), a natural antimicrobial peptide from a plant. The dual activity of these peptides also helps address the intersection between cancer and infections, where chronic infections and their treatment can lead to the induction of cancer progression, and inversely, the weakened immune system from cancer treatments herald opportunistic infections. In the present study, the fifteen amino acid length fragment of CyO2 (Uniprot ID: P58434) was mutated with lysine residues, resulting in increased helical propensity and stability. Our results showed that a novel fragment T2.2 (double lysine substitution) peptide had the most stable physicochemical properties (amphipathicity, charge, hydrophobicity, half-life, and molecular weight) and highest biological activities (anticancer and antimicrobial) among the CyO2-derived peptides. This highlighted the importance of lysine residue in developing stable therapeutic peptides with low hemolytic activity. The molecular docking study indicated the potential of T2.2 to induce extrinsic apoptosis by binding to the death domain of the Fas receptor. Our results indicated that the short T2.2 peptide derived from CyO2 could be considered a potential therapeutic agent in anticancer and antimicrobial treatments. Furthermore, *in vitro* and *in vivo* studies are essential to confirm the predictions obtained by the *in silico* analysis.

Keywords: cancer; antimicrobial; peptide; anticancer; *in silico*; Cycloviolacin O2

1. Introduction

Cancer is one of the leading causes of morbidity and mortality across the globe, with an incidence rate of 27.5 million new cases per annum expected by 2040, which is a sharp increase from the 20 million new cases reported worldwide in 2020 [1]. The current gold standards of cancer therapy are still plagued by low success rates and severe side effects, incurring an urgent need for research into developing novel methods of cancer treatment that bypass these limitations. Furthermore, with the rise in multidrug-resistant infections, there is a

need to design new antimicrobial therapies. Particularly in relation to cancer, multi-resistant infections are a cause for concern due to the synergistic nature of cancer and infections. Aggressive cancer therapies such as radiotherapy, chemotherapy, and surgical resections can leave the patients more prone to opportunistic infections [2].

Additionally, infections are associated with 15% to 20% of cancers worldwide [3], as certain infections can lead to immune suppression, long-term inflammation, and genetic alterations, raising cancer risk. An emerging technique that can address the aforementioned concerns involves molecular targeting cancer cells using naturally occurring peptides such as plant antimicrobial peptides (AMPs) with innate anticancer potentials [4,5]. However, some of the challenges that need to be overcome regarding these peptides are their short half-lives, low stability, susceptibility to *in vivo* cleavage by proteases, and low bioavailability [6,7]. To achieve this, *in silico* predictive models can be used to screen for peptides with anticancer capacity and to predict the mutations required to be introduced to modify the peptides' physicochemical parameters for the rational design of anticancer peptides (ACPs) [8,9]. *In silico* analysis also allows for the simultaneous evaluation of large quantities of data in a cost and time efficient manner to narrow down and identify the best molecules to carry forward to the *in vitro* analysis stages [10].

Plant AMPs are ideal candidates for designing ACPs with dual antimicrobial and anticancer properties as they are easy to obtain and modify and less likely to induce resistance [11]. These peptides can also often kill multidrug-resistant cells, active tumor cells, opportunistic infections, and even slow-growing cancer cells [12,13]. They also have minimal drug-drug interactions, are less immunogenic, and are less likely to accumulate in certain organs, which minimizes their toxicity [14,15]. These peptides have diverse activities depending on their amino acid composition and structural conformations. The selectivity of most AMPs towards bacterial cells and cancer cells seems to be due to the net negative charge of their membranes, which is in contrast to normal mammalian cells that are typically zwitterionic, thus, allowing selective targeting by cationic peptides [13,16].

Cycloviolacin O2 (CyO2) is a cyclotide AMP with a particular interest in cancer treatment due to its selective toxicity towards cancer cells [17]. It has 30 amino acids in its primary structure with an overall charge of +2, giving it selectivity towards bacterial cell walls and cancer cell membranes. The potent antimicrobial activity of CyO2 was demonstrated by Pranting *et al*.[18] where CyO2 had the highest antimicrobial activity against gram-negative microbes (*S. enterica*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa*), when compared to other tested AMPs. However, CyO2 was not as effective against *S. aureus*, likely due to the thick, protective peptidoglycan layer of gram-positive cells. The anticancer mechanism of CyO2 was validated by a study that showed that the morphological change observed in cancer cells (human lymphoma) *in vitro* was necrotic death [19]. However, it has a low therapeutic index due to its weak positivity, which reduces specificity for microbial cells and cancer cells [20], and due to its necrotic anticancer mechanism, which can induce inflammation [17,21]. Identifying bioactive fragments from this sequence and altering their physicochemical properties using predictive models could optimize their anticancer potential and eliminate these limitations [22]. Therefore, we utilized several *in silico* methods to design more potent, less cytotoxic anticancer and antimicrobial peptides from CyO2.

2. Materials and Methods

2.1. Prediction of physicochemical characteristics of the peptides.

The peptide sequence for CyO2 was retrieved from UniProt via the accession number P58434. The physicochemical and anticancer properties of the template T2.0 fragment (15 amino acids in length) and its derivatives (T2.1, T2.2, and T2.3) were predicted using three available anticancer predictive servers: AntiCP (https://webs.iiitd.edu.in/raghava/anticp2/), iACP (http://lin.uestc.edu.cn/server/iACP), ACPred (http://codes.bio/acpred/). The single amino acid substitution produced T2.1 peptide, where the serine (S), the polar, uncharged residue at location 1 of T2.0, was replaced with positive-charged lysine (K) residue. Similarly, double amino acid substitution produced T2.2 peptide where the two serine (S) polar uncharged residues at locations 1 and 2 of T2.0 were replaced with lysine (K) residues. The fifth amino acid (glycine, G) in T2.2 was replaced with a lysine residue, producing T2.3 (triple amino acid substitution). The following online tools were used to assess their physicochemical properties and activities: AntiCP's Peptide Design function was used to predict the amphipathicity, molecular weight, hydrophilicity, and hydrophobicity of the peptides while DBAASP (https://dbaasp.org/) was used to predict their propensity to *in vitro* aggregate. PLifePred (https://webs.iiitd.edu.in/raghava/plifepred/) was used to predict the half-life of the selected peptides in seconds, and PSSP-MVIRT (http://server.malab.cn/PSSP-MVIRT) and PEP2D (https://webs.iiitd.edu.in/raghava/pep2d/) were used to predict the secondary structure of peptides in terms of random coils, alpha helix, and beta sheets. Toxicity and hemolytic activity were predicted using ToxinPred (http://crdd.osdd.net/raghava/toxinpred/) and HemoPI (https://webs.iiitd.edu.in/raghava/hemopi/), respectively. ClassAMP (http://www.bicnirrh.res.in/classamp/) and CAMPR3 (http://www.camp.bicnirrh.res.in/) were used for antimicrobial predictions, and C2Pred (http://lin-group.cn/server/C2Pred) and CellPPD-MOD (https://webs.iiitd.edu.in/raghava/cellppdmod/) were used for cell penetration prediction.

2.2. Docking of peptides with the anticancer molecular target, Fas receptor.

In the case of low cell penetration propensity, the ability of the peptide to potentially induce apoptosis through an extrinsic method was evaluated by docking the selected peptides with an extracellular death domain of the receptor Fas/CD95. The PDB of the receptor was obtained through a protein bank as a crystal structure of Fas receptor extracellular domain in complex with Fab E09 (PDB ID: 3TJE). The Discovery Studio Visualiser software was used to prepare the receptor for docking by removing water molecules and adding polar hydrogens and kollman charges to the structure. Afterward, HPEPDOCK (http://huanglab.phys.hust.edu.cn/hpepdock/) and HDOCK (http://hdock.phys.hust.edu.cn/) were used to dock the receptor with the selected peptides and, as a positive control, the receptor was also bound with a known ligand (Crystal structure of a human FasL mutant, PDB ID: 5L19). In addition to this physiological Fas ligand (FasL), KT2 (NGVQPKYKWWKWWKKWW), a leucrocin peptide derived from crocodile leukocyte extract, was used as a control as well [23]. KT2 is a published peptide that has been validated *in vitro* to induce apoptosis in HeLa cells and colorectal and cervical cancer cells [24]. In this study, local docking was used where the binding residues of the Fas death domain were defined as per the report by Starling *et al*. [25], where they identified the essential residues for ligand

binding to be residues 86 and 87 on domain 2. This was input into the servers as 86:F and 87:F for the two Arg residues on chain F. HDOCK also gave the receptor and ligand interface residues for each binding. The 3D models generated by HDOCK for all bindings were downloaded, and input into the LigPlus software, and its DIMPLOT function was used to generate receptor-ligand interactions classified into different types of bonds.

3. Results and Discussion

3.1. Physicochemical and anticancer properties of CyO2 and its derivative peptides.

The template peptide, T2.0 was selected due to the concentration of the Lysine residues in the fragment, which resulted in high anticancer predictions. The template was then sequentially mutated with lysine to produce three mutated peptides; T2.1 (single mutation), T2.2 (double mutation), and T2.3 (triple mutation) (Table 1). The introduction of Ks (lysine) to replace S (serine) and G (glycine) is a favorable approach as Ks are relatively more hydrophilic due to their polarity and positive charge in contrast with S, which is merely polar but uncharged, and G, which is nonpolar and hydrophobic [26,27]. This high frequency of K is a pattern observed for ACPs in a study by Agrawal *et al*. [28]. *In vitro* studies by Pranting *et al*. [18] also highlighted the importance of K in CyO2, as modifying the K residues led to a 7-fold decrease in anticancer potency. Besides hydrophilicity, lysine is also much more favorable as it is less likely to cause hemolysis and has a side chain that is much more hydrophobic than arginine [29]. Therefore, by fulfilling both criteria of having sufficient levels of hydrophobicity and cationic, lysine best qualifies as the amino acid of choice when introducing substitutions to potential ACPs [28,30]. Lastly, the high content of lysine permits a possible snorkeling mechanism of action facilitated by the insertion of lysine's long hydrophobic chain into the cell membrane core leading to membrane disruption [31].

Table 1. Prediction of physicochemical and anticancer properties of CyO2 and its derivative peptides.

Peptide	Sequence	Physicochemical prediction					Anticancer prediction		
		Cationicity	Amphipathicity	Hydrophobicity	Hydrophilicity	Propensity to *in vitro* aggregate	AbtiCP	ACPred	iACP
CyO2	GIPCGESCVWIPCISSAIGC SCKSKVCYRN	+2	0.37	-0.04	-0.29	0.81	0.780	0.998	0.998
T2.0	SSAIGCSCKSKVCYR	+3	0.65	-0.21	0.07	0.00	1.010	0.996	0.563
T2.1	KSAIGCSCKSKVCYR	+4	0.90	-0.27	0.25	0.00	1.080	0.998	0.555
T2.2	KKAIGCSCKSKVCYR	+5	1.14	-0.32	0.43	0.00	1.040	0.999	0.586
T2.3	KKAIKCSCKSKVCYR	+6	1.39	-0.41	0.63	0.00	0.900	0.999	0.581

High amphipathicity and cationicity are associated with increased anticancer properties [32-34]. This trend was observed in this study as the anticancer potential was higher in all of the mutated peptides which are more cationic and amphipathic than the original CyO2 peptide according to AntiCP scores, as demonstrated in Table 1. Amphipathic nature is almost ubiquitously seen in peptides with dual anticancer and antimicrobial activities [34]. In the context of cationicity, the mutated peptides had higher cationicity than the original peptide,

ranging between +3 to +6, which fell within the optimal range of +2 to +9, thus, will have higher selectivity as they will be electrostatically attracted to the cancer cell and bacterial membranes' negative charge without compromising the peptides' secondary structure [35]. When the charge is higher than +9, it can lead to increased hemolytic activity reducing its clinical use as an anticancer agent. Further, the high charge can compromise the secondary structure due to repulsion between residues or due to the drastic increase in electrostatic attraction to negative molecules [36,37]. In a study conducted by Yang *et al*. [38], an AMP named temporin-1CEa was systematically altered by substituting its neutral acid residues with the positive amino acid residue, lysine. They created four analogs which increased the cationicity from +3 in the original peptide to +7. The IC_{50} values in this study had a tendency to decrease as the cationicity increased, indicating higher anticancer activity.

The original peptide, CyO2 had a positive charge of only +2, which is just at the lower limit of the range of ideal cationicity for increased anticancer potential [35]. This is likely a factor in CyO2's relatively lower anticancer activity, as predicted by AntiCP, as shown in Table 1. However, the iACP server predicted CyO2 to have a significantly higher anticancer potential, at 0.998, than its derivative peptides, which ranged between 0.555 and 0.586, as demonstrated in Table 1. This discrepancy in anticancer potential might be attributed to CyO2's higher hydrophobicity. Typically, hydrophobicity is associated with cell cytotoxicity and lytic activity as it leads to the enhanced ability of peptides to penetrate deeper into the membrane's hydrophobic core and consequently permeabilize the membranes [20,34]. Therefore, while increased cationicity and amphipathicity can supplement their anticancer potential, the peptides' propensity to destroy cancer cells through necrotic mechanisms can be hindered by decreasing hydrophobicity [20]. This argument is further supported when Yang *et al*. [39] found that high anticancer therapeutic potential can be derived by increasing cationicity while keeping the hydrophobicity at a low to moderate level. In the present study, the hydrophobicity of the peptides decreased with each mutation, starting from -0.04 in CyO2 and ultimately decreasing to -0.41 in T2.3, as shown in Table 1. This might explain why the highest anticancer score from AntiCP was achieved not by the most cationic peptide (+6), T2.3, as it had an extremely low hydrophobicity (-0.41), but by the peptide T2.1 which simultaneously had a high cationicity of +4 along with an almost 1.5 times as high hydrophobicity at -0.27. After T2.1, the peptides T2.2 and T2.3 had a downward trend in anticancer scores predicted by AntiCP, which can be ascribed to their decrease in hydrophobicity as a major factor.

With each mutation introduced to the peptide, its amphipathicity increased as well, starting from 0.37, as observed in CyO2, and increasing up to 1.39, as observed in T2.3, in Table 1. This increase in amphipathicity is expected to increase the likelihood of self-aggregation. However, the self-aggregation predictions only yielded one positive score for CyO2 at 0.80, while all the mutated peptides had a score of 0. Despite these predictions, given the high amphipathicity of all mutated peptides, their effect on aggregation or self-assembly can increase their specificity to negatively charged cancer cell and bacterial membranes, thus increasing both antimicrobial and anti-cancer efficacy [40,41]. Further benefits of the increased amphipathicity are indicated in a study by Edwards *et al*. [42], which evaluated the relationship between the amphipathicity of different beta-hairpin peptides and their antimicrobial activities. They found that compared to hydrophobicity or cationicity alone, overall amphipathicity was more strongly correlated with anticancer activity. From the studied beta-hairpin peptides, tachyplacin-1 had an 8-fold higher amphipathicity than gomesin and exhibited much more broad-spectrum and potent antimicrobial activity against a panel of bacteria and fungi as well.

Another aspect for consideration is the extent of the membrane penetration potential of the peptides. It can be observed in Table 2 that the penetrative cell potential for all peptides was low, with a range of -1.165 to -0.121 in the predictions using CellPPD-MOD and with a range of 0.065 to 0.195 in the predictions using C2Pred. Hydrophobicity was reported to play a role in membrane penetration [38]. As all the peptides, including CyO2, had negative values of hydrophobicity, this might explain the low cell penetrative scores given by both servers for all the peptides. Further lack of penetrative cell property can be attributed to the increase in lysine residues which lack the guanidine headgroups that give cell penetrative potential to other cationic peptides rich in arginine instead [43].

Table 2. Cell penetrative potential of CyO2 and its derivative peptides.

Peptides	CellPPD-MOD	C2Pred
CyO2	-1.165	0.065
T2.0	-0.974	0.146
T2.1	-0.981	0.146
T2.2	-0.641	0.151
T2.3	-0.121	0.195

The secondary structure assumed by the peptide is also a crucial consideration, as the conformations assumed affect cell surface interactions, influencing the peptide's antimicrobial and anticancer efficacy [44]. In the present study, secondary structure predictions yielded inconsistent results. The predictions generated using the PSSP-MVIRT server yielded high occurrences of helices, which increase even more with each subsequent mutation which can lead to increased anticancer potential. This factor was observed in a study by Wang *et al*. [45] which reported that a peptide polybia-MPI had selective inhibitory effects on prostate and bladder cancer cell proliferation and that its alpha-helical structure was significant in achieving its anticancer effect. In contrast to the increasing helicity predicted by PSSP-MVIRT, the predictions generated using PEP2D indicated a high propensity for forming beta sheets with no prediction of helices, even with the introduction of mutations, as shown in Table 3. It has been hypothesized that with increased hydrophobicity, the helical propensity of peptides will increase [20]. However, according to the secondary structure predictions by PSSP-MVIRT, helical propensity increased with each mutation despite the decrease in hydrophobicity from T2.0 to T2.3. This might be because, despite the predicted decrease in hydrophobicity with each mutation, the number of hydrophobic residues in the unmutated T2.0 template, which was 7 (A, I, G, 3Cs, V) decreased to 6, only with the third mutation which replaced the hydrophobic G residue which is weakly hydrophobic, thus, overall hydrophobicity might have been mostly preserved [26]. Furthermore, considering the different secondary structure predictions by PSSP-MVIRT versus Pep2D, PSSP-MVIRT's predictions hold more validity according to several factors. Firstly, serine and glycine, two of the lost residues to the mutation cycles, are considered hindering residues to helix formation [46,47], therefore, their removal might be the cause for the increased helicity predicted. Additionally, as predicted by Pep2D, beta-sheet peptides often have two or more disulfide bridges, which is not possible in the selected peptides as they only have three cysteines. Another property that can also influence secondary structure is the length of the peptides; shorter peptides are more likely to form alpha helices [48], as predicted by PSSPMVIRT. Taking all the observations together, it can be inferred that these peptides with increasing mutations introduced would have an increased propensity to form helices. Cationic α-helices have been demonstrated to exhibit relatively higher specificity and

efficacy for cancer cells [49,50]. Hence, with each mutation introduced to the peptide, the higher the potential anti-cancer efficacy the peptides have.

Table 3. Secondary structure prediction of CyO2 and its derivative peptides.

Peptide	PSSP-MVIRT C = coils, H = helices, E = beta sheets	PEP2D C = coils, H = helices, E = beta sheets
CyO2	--	CEEEECCCCCCCCCHCHHCCEEECCEEEEC
T2.0	CCHHCCCCHCHHCHH	CCCCCCEEECCEECC
T2.1	HCHHCCCCHCHHCHH	CCCCCCEEECCEECC
T2.2	HHHHCCCCHCHHCHH	CCCCCCEEECCEECC
T2.3	HHHHHCCCHCHHCHH	CCCCCCEEECCEECC

Beyond the physicochemical properties and secondary structures of the peptides, the half-life and molecular weight of the peptides were also investigated. It can be seen in Table 4 that all the selected peptides had a higher half-life than CyO2. However, CyO2 is characteristic of its high stability due to its cysteine knot motif cyclic structure. This cyclic structure and increased stability are ascribed to its cysteine residues. CyO2 can form 3 disulfide bonds due to its 6 C residues, while the selected peptides can form 1 disulfide bond with their 3 C residues. But CyO2 is relatively longer compared to the 4 selected peptides. Shorter fragments are more likely to be less immunogenic and more stable. This might be the reason for the significant increase in half-life predictions from the 30 amino acid length in CyO2 to the selected peptides of half this length, as these shorter fragments are more biologically stable. With the increasing stability through longer half-life predicted for the selected peptides, this trend was slightly broken by the 3rd mutation, where T2KKK had a lower half-life of 838.91 compared to T2KK at 862.61. This might be attributed to the increased cationicity, which, after a certain point, can lead to decreased stability due to repulsion between the similarly charged residues [47]. According to studies by Mathur *et al*. 2017 [50], higher proportions of small residues such as G, A, and I contribute to longer half-life, while neutral residues like S shorten the half-life. Therefore, another explanation for the slight decrease in half-life after the third mutation could be because of the substitution of the small G residue, which promotes a longer half-life with the large K residue. Although the stability predictions increased compared to CyO2, the highest half-life achieved is still relatively short at 862.61 seconds. This implies that maintaining the peptide concentration at therapeutic levels would be difficult, hence requiring the use of half-life prolonging strategies [51,52].

Table 4. Stability and molecular weight prediction of CyO2 and its derivative peptides.

Peptide	Half Life (Seconds) (via PLifePred)	Molecular weight (via AntiCP)
CyO2	690.11	3165.18
T2.0	707.81	1592.07
T2.1	760.41	1633.17
T2.2	862.61	1674.27
T2.3	838.91	1745.39

Considering toxicity predictions, ToxinPred predicted that all of the peptides are non-toxins, with SVM scores ranging from -1.15 to -0.38 (Table 5). It is worth noting, however, that CyO2 had the lowest SVM score, at -1.15, while its CyO2-derivative peptides had SVM scores ranging from -0.38 to -0.68. This indicates that CyO2 is less likely to exert toxin effects

than its derivative peptides. The significantly lower toxicity score for CyO2 might result from its higher molecular weight, as the larger size can interfere with membrane interaction [53]. There were contradictory trends in results generated by ToxinPred and HemoPI as the toxicity predictions from ToxinPred showed increasing trends, with SVM scores increasing from CyO2 (-1.15) to T2.3 (-0.38).

In contrast, the hemolytic activity decreased from CyO2 (0.83) to T2.3 (0.49). This contrast conflicts with studies that report that hemolytic activity is a good indicator of toxicity towards higher eukaryotic cells. A possible explanation could be that, as previously discussed, helicity increased while preserving most of the hydrophobic residues in the peptides with each mutation. This increase in helicity concurs with the toxicity scores as these two properties are associated with each other [20]. However, the direct hydrophobic predictions decreased from CyO2 to T2.3, which could explain the decrease in hemolytic activity scores in this direction. But this justification falls short when considering that both ToxinPred and HemoPI use the same database to predict hydrophobicity, thus, giving the same results. This indicates that the results from ToxinPred where toxicity was shown to increase while hydrophobicity decreased, contradicting the existing literature. But it must also be acknowledged that this trend was only observed for such a small sample size of only 5 peptides studied in this paper which can lead to false conclusions in terms of these trends. A larger sample size of peptides would be needed to validate these arguments further.

Table 5. Prediction of toxicity and hemolytic activity of CyO2 and its derivative peptides.

Peptide	ToxinPred	HemoPI
CyO2	-1.15	0.83
T2.0	-0.49	0.51
T2.1	-0.68	0.51
T2.2	-0.43	0.49
T2.3	-0.38	0.49

The innately antimicrobial peptide, CyO2, had higher antimicrobial potential scores in both servers compared to the 4 CyO2-derived short peptides (Table 6). This could be the result of prioritizing anticancer activity in the design process, in which physicochemical properties, such as amphipathicity, cationicity, and hydrophobicity, were modified to increase the specificity of cancer cells. However, the two physicochemical properties used in the selection, amphipathicity, and cationicity, are also important for antimicrobial activity [54-56]. The selected peptides had much lower hydrophobicity than CyO2, likely the main contributor to their reduced AMP potential. The mutations in the first two cycles also removed serine residues associated with AMPs [57].

Table 6. Prediction of antimicrobial activity of CyO2 and its derivative peptides.

		CampR3		ClassAMP	
Peptide	SVM	Random Forest Classifier	Discriminant Analysis	Prediction	Probability
CyO2	0.997	0.995	0.996	Antifungal	0.983
T2.0	0.527	0.497	0.476	Antiviral	0.971
T2.1	0.814	0.572	0.641	Antiviral	0.976
T2.2	0.771	0.699	0.792	Antiviral	0.958
T2.3	0.371	0.779	0.835	Antifungal	0.962

The anticancer mechanism of action for the selected peptides is most likely a necrotic cell membrane disruptive mechanism, perhaps a snorkeling method, as peptides rich in K and

R often exhibit [31]. The high proportion of hydrophobic residues in the selected peptides further supports the necrotic mechanism. This proportion is observed in all CyO2-derivative peptides, from T2.0 to T2.3, where they had 3 highly hydrophobic residues, I, A, and V, along with 4 moderately or weakly hydrophobic residues, three Cs, and one G [26]. The mutations that followed T2.0 substituted the neutral S residues in the first two cycles and the weakly hydrophobic residue, G, in the third cycle. Helicity was also increased, which can further facilitate cell membrane permeability. However, the CyO2-derivative peptides exhibited low penetrative potentials, leading to a lower likelihood of necrotic mechanisms. Therefore, the possibility of apoptosis induction through an extrinsic pathway is also considered and tested in this study. To test the possibility of the peptides in exerting anti-cancer effects via anextrinsic pathway, the interaction of the peptides with the Fas receptor was tested.

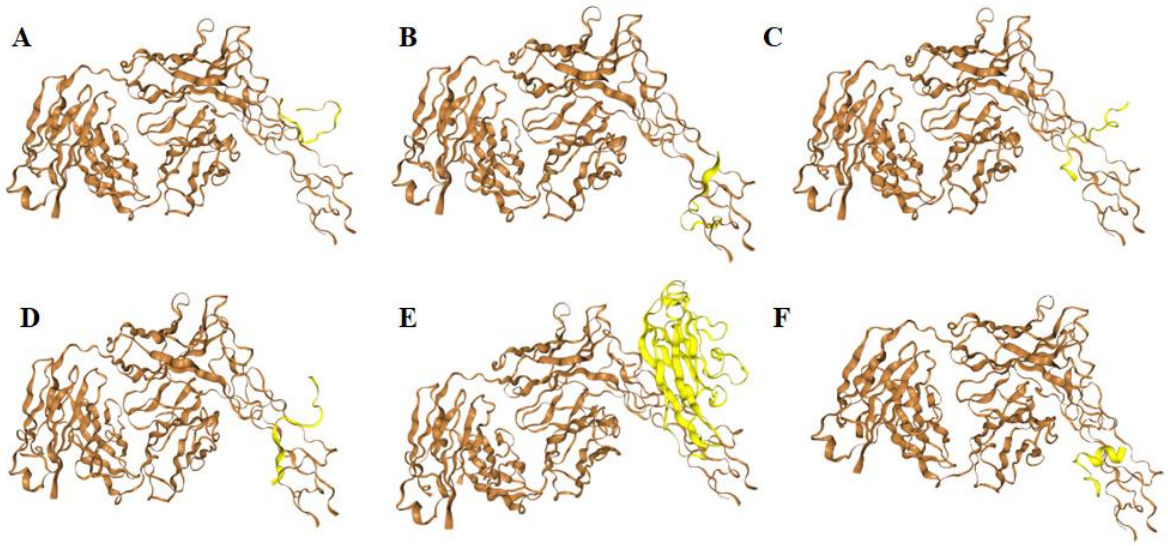

Figure 1. Molecular interaction of ligand (yellow) and receptor (Fas receptor extracellular domain, brown) in 3D interaction analysis. (**A**) T2.0 (template peptide), (**B**) T2.1 (single mutation), (**C**) T2.2 (double mutations), (**D**) T2.3 (triple mutations), and € FasL, and (**F**) KT2 (as positive control)

Figure 1 demonstrates the molecular interaction of CyO2, its derivative peptides, FasL and KT2, targeting the active site of Fas death domain [58]. Based on Table 7, the docking scores between Fas extracellular domain and the peptides resulted in the highest score being -199.96 by T2.2 while the natural Fas ligand scored - 225.13 in HDOCK, a difference of just 25 units, and the peptide, KT2 scored -253.96, a difference of 54 units compared to T2KK indicating an even stronger binding potential by KT2 to Fas than any of the derivative peptides or FasL according to HDOCK. However, these results might be skewed by the binding parameters and database biases. Therefore, they must be further validated using *in vitro* tests. Besides the natural ligand and the positive control, T2.2, one of the highest overall scoring anticancer peptides for all three anticancer prediction servers, also had the highest docking scores.

Table 7. Docking scores between the peptides and Fas extracellular domain.

Ligands	HDOCK	HPEPDOCK
T2.0	-186.02	-175.320
T2.1	-180.74	-175.051
T2.2	-199.96	-169.435
T2.3	-177.97	-167.483
FasL (natural ligand to Fas)	-225.13	--
KT2 (as positive control)	-253.96	--

None of the peptides exhibited salt bridges in the defined binding region, according to LigPlus (Figure 2); salt bridges are more favorable as they are stronger than hydrogen bonds. The HDOCK's interface residue predictions (Figure 3) did indicate a few possible salt bridges, which can only be deduced by the amino acids involved, and the Angstrom values as the server does not indicate the type of interaction. For example, some of the interface residues predicted by HDOCK for Fas involved aspartate and glutamate, which can form salt bridges with the cationic lysines or arginine in the derived peptides. In T2.0 and Fas interaction, Asp at 39F of the Fas domain interfaced with Arg on the 15th position of T2.0 with an Angstrom of 2.59 which is a reasonable distance for salt bridges. T2.1 and T2.2 also had potential interactions with Fas for salt bridges with Glu and Lys (3.11 A) and Asp and Lys (3.50 A), which might be stronger due to the higher positive charge of lysine; however, affinity can be hindered by the longer distance [59].

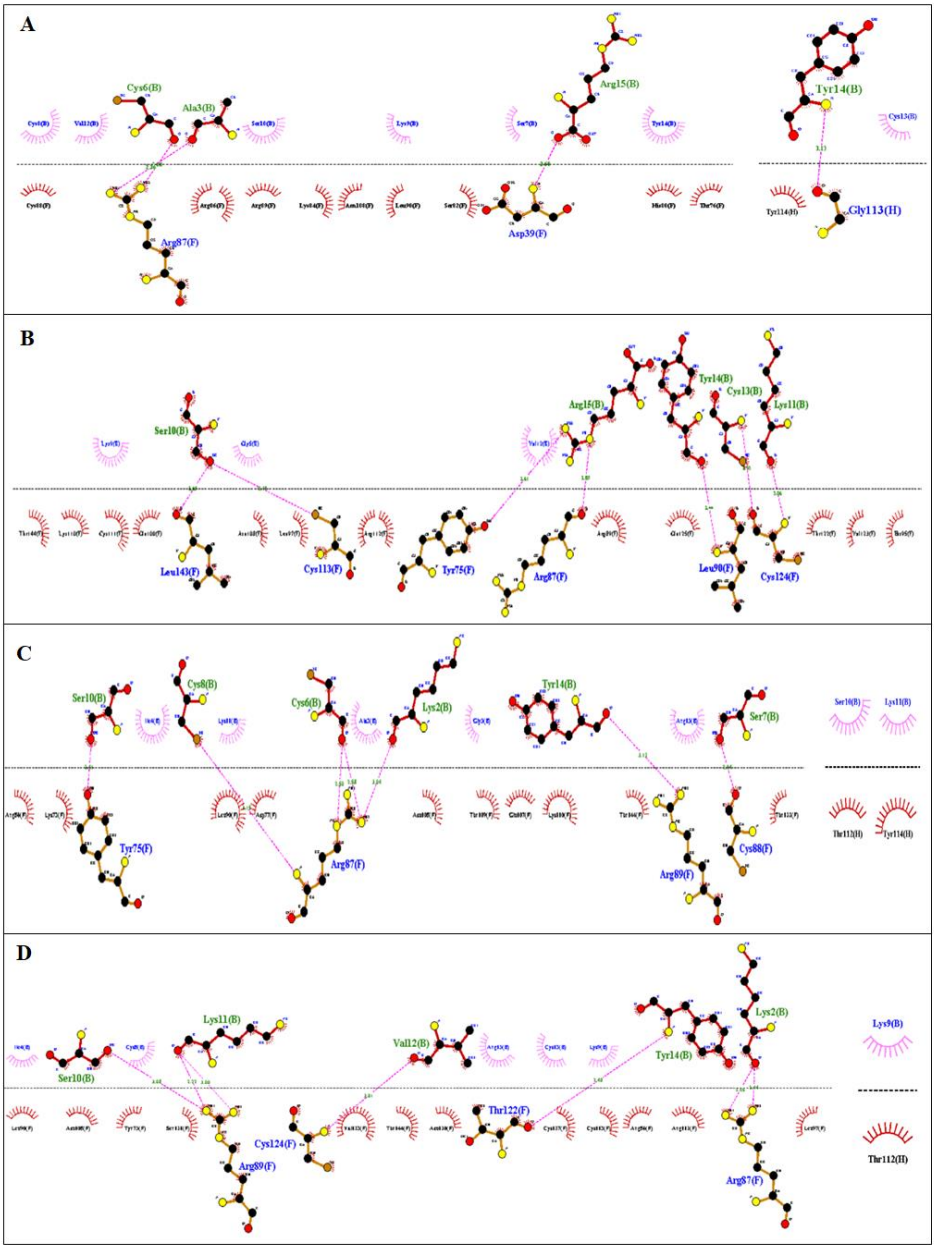

Figure 2. Receptor-peptide interactions predicted by LigPlus. (**A**) T2.0 (template peptide), (**B**) T2.1 (single mutation), (**C**) T2.2 (double mutations), (**D**) T2.3 (triple mutations), and (**E**) FasL. Atoms are colored yellow for nitrogen, red for oxygen, and black for carbon. Hydrophobic interactions are brick red for the receptor (bottom) and pink for the ligand (top), and hydrogen bonds are lilac for both.

In terms of hydrogen bonds, they usually have a distance between 2.6 to 3.3 Angstrom, while hydrophobic interactions have a range between 3.3 and 4.0 [60]. Considering these parameters, T2.0 had 14 interactions that fell within the hydrogen bonding range, T2.1 had 21, T2.2 had 16, and T2.3 had 19. And considering the range for hydrophobic interactions, T2.0 had 13, T2.1 had 14, T2.2 had 17, and T2.3 had 9 interfaces with Angstroms between 3.3 and 4.0. These predictions by HDOCK indicate that an overall stronger attraction must be exhibited by T2.1 followed by T2.2. However, the actual HDOCK results indicate scores in ascending order T2.2 > T2.0 > T2.1 > T2.3. This might be the result of the cut-off point used for interfaces in HDOCK being 5 Angstroms. The values could also be skewed by the distances within the defined range. From these results, it can be deduced that T2.2 and T2.1 can potentially be valuable Fas agonists to its external death domain, rendering apoptotic and necrotic mechanisms of anticancer activity. Other AMPs have previously been observed to induce apoptosis and necrosis *in vitro*, such as HNP [61], LfcinB [62], and MG2A. [63].

Fas - T2			Fas - T2K			Fas - T2KK			Fas - T2KKK		
112H	12B	4.838	75F	15B	2.609	112H	10B	3.327	112H	9B	3.778
112H	13B	4.386	86F	15B	4.298	112H	11B	4.751	113H	9B	4.691
113H	13B	3.598	87F	15B	2.850	114H	10B	3.284	75F	10B	3.261
113H	14B	2.906	88F	15B	4.121	114H	11B	3.727	86F	9B	3.790
114H	14B	3.340	89F	12B	4.053	72F	4B	4.314	87F	1B	3.139
115H	14B	4.218	89F	13B	4.336	73F	3B	3.395	87F	2B	2.442
39F	15B	2.598	89F	14B	3.224	73F	4B	3.202	87F	3B	4.226
73F	8B	4.574	89F	15B	3.064	75F	8B	4.440	87F	4B	4.203
75F	10B	3.525	90F	14B	1.825	75F	9B	3.620	88F	4B	4.099
76F	14B	3.591	90F	15B	3.629	75F	10B	2.359	89F	4B	4.936
78F	14B	3.791	91F	14B	3.955	75F	11B	4.380	89F	8B	3.216
79F	14B	4.984	95F	14B	3.770	77F	11B	3.528	89F	10B	3.082
80F	14B	3.090	97F	12B	2.230	78F	11B	4.905	89F	11B	2.219
82F	14B	2.818	97F	13B	3.651	86F	8B	4.040	89F	12B	2.922
82F	15B	4.380	97F	14B	3.998	86F	10B	2.831	89F	13B	4.992
84F	1B	4.723	100F	8B	4.065	86F	11B	4.747	90F	4B	3.747
84F	14B	3.543	100F	9B	3.109	87F	2B	3.042	90F	8B	4.674
85F	1B	4.053	100F	10B	4.019	87F	3B	3.467	97F	14B	1.990
85F	12B	3.928	101F	9B	4.144	87F	4B	3.392	108F	10B	2.732
86F	1B	4.877	108F	15B	3.466	87F	5B	3.872	108F	11B	4.906
86F	10B	4.333	110F	9B	2.717	87F	6B	2.505	111F	12B	4.367
86F	11B	4.977	110F	10B	4.068	87F	7B	4.366	111F	13B	4.919
86F	12B	2.222	110F	11B	4.123	87F	8B	3.146	111F	14B	3.976
86F	13B	3.968	111F	10B	2.618	87F	10B	4.447	112F	14B	3.225
86F	14B	3.078	111F	11B	4.516	88F	4B	4.247	113F	14B	3.108
87F	1B	4.654	111F	12B	3.169	88F	6B	4.894	119F	14B	4.419
87F	2B	4.331	111F	13B	4.802	88F	7B	2.940	119F	15B	4.834
87F	3B	2.359	111F	14B	4.515	89F	7B	3.213	120F	15B	3.030
87F	4B	4.547	112F	5B	4.628	89F	8B	4.528	121F	14B	4.054
87F	6B	3.075	112F	10B	3.262	89F	9B	3.834	121F	15B	3.555
87F	7B	3.627	112F	12B	3.416	89F	11B	4.462	122F	12B	4.003
87F	8B	3.578	113F	10B	3.170	89F	14B	2.871	122F	13B	2.604
87F	9B	3.159	113F	12B	3.681	89F	15B	3.917	122F	14B	2.484
87F	10B	2.944	115F	4B	4.078	90F	4B	3.473	122F	15B	2.948
87F	11B	4.779	119F	10B	4.391	90F	5B	3.626	123F	11B	3.007
87F	12B	3.269	122F	11B	2.924	90F	6B	4.950	123F	12B	2.850
88F	8B	1.763	123F	11B	3.581	90F	7B	3.313	123F	13B	3.885
88F	9B	4.239	123F	13B	3.295	101F	14B	3.900	123F	14B	4.587
88F	10B	4.318	124F	11B	3.063	107F	14B	3.377	124F	12B	3.014
89F	8B	3.934	124F	12B	3.184	108F	8B	4.397	124F	13B	4.624
89F	9B	3.942	124F	13B	2.931	108F	9B	3.205	124F	14B	2.782
89F	10B	3.011	124F	14B	4.880	108F	10B	4.627	127F	14B	3.537
90F	7B	4.695	125F	12B	4.958	108F	14B	2.561	143F	14B	4.835
90F	8B	3.358	125F	13B	3.423	109F	14B	3.095	144F	14B	3.515
108F	10B	2.589	125F	14B	2.693	110F	14B	2.675	144F	15B	3.729
			126F	13B	4.736	110F	15B	3.084			
			126F	14B	4.693	111F	15B	4.298			
			127F	10B	4.591	119F	15B	4.562			
			127F	12B	3.918	120F	15B	4.478			
			142F	7B	3.937	121F	15B	3.635			
			143F	4B	4.760	122F	15B	3.172			
			143F	5B	2.613	123F	9B	4.241			
			143F	6B	3.993	123F	12B	4.674			
			143F	10B	2.647	144F	15B	3.785			
			144F	7B	4.747						
			144F	9B	2.815						
			144F	10B	3.429						

Figure 3. Receptor-ligand interface residue pairs predicted by HDOCK (By considering interactions with less than 5 Angstroms).

The design process of peptides for dual anticancer and antimicrobial potential should implement antimicrobial prediction servers in concert with the anticancer prediction servers. Omission of which from the selection and design process of the peptides can lead to, as in this study, relatively lower antimicrobial predictions as anticancer properties were prioritized, despite the correlating properties for anticancer and antimicrobial activities. Furthermore, designing peptides to target specific receptors can be optimized by studying the binding site of the receptor and its natural ligands to aim for binding mimicry [64]. *In vitro* tests are also vital in validating the in-silico predictions obtained in this study and confirming the hypotheses as peptides can confer different conformations and exhibit different activities in a real biological environment compared to the predictions using bioinformatics servers which can depend on the quality of the datasets used to train their algorithms [65,66]. This can lead to algorithm issues with regard to discrimination between peptides with similar compositions but different activities. Further refining the design process of ACPs to target more specific cancer tumorigenesis steps or to have more specific functions, such as necrotic mechanism, penetrative cell potential, or binding to a specific receptor, can lead to the derivation of more therapeutic ACPs. And finally, a larger sample size of peptides can be used in future work better to understand the patterns of physicochemical properties and activities.

4. Conclusions

AMPs are a promising candidate to therapeutically address cancer and infections, which are two leading causes of morbidity and mortality across the globe, having synergistic properties to exacerbate one another. However, there is a need to optimize these molecules by improving their stability and cellular uptake, reducing toxicity, and increasing bioavailability before they can reliably challenge existing therapeutic methods. This study aimed to optimize the dual activities of an existing AMP, CyO2, through the use of fragmentation and amino acid substitution techniques. These CyO2-derived peptides all had mutations that inserted lysine into the sequence, which subsequently increased their cationicty and amphipathicity, thereby increasing their potential for higher selectivity towards bacteria and cancer cells. The shorter fragment sizes also gave them increased stability predictions, even compared to the characteristically stable CyO2. However, compared to the innately antimicrobial CyO2, all the peptides had lower antimicrobial predictions, likely due to their lower hydrophobicity. Low hydrophobicity also translated into lower hemolytic activity, indicating that the peptides will be less cytotoxic to normal cells. Overall anticancer predictions from two of the three servers used yielded higher anticancer scores for the derived peptides than the original CyO2 peptide, which can be attributed to their higher amphipathicity and cationicity. The most likely mechanism of action of these peptides against cancer cells is a snorkeling mechanism often exhibited by peptides with high lysine content. When the Fas receptor-mediated apoptotic pathway was considered, the results suggested the potential for apoptosis and necrosis, particularly by the peptide T2.2. In conclusion, the present study has identified CyO2-derived peptides, which may be more potent in dual anticancer and antimicrobial activities than the original peptide.

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Conflicts of Interest

The authors declare no conflict of interest.

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