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RESEARCH ARTICLE

Optimizing Anticancer Peptides Through Structural Activity Relationships as a Breast Cancer Treatment Approach

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ABSTRACT: Breast cancer persistently ranks as the second most prevalent cause of cancer fatalities among the female population worldwide, underscoring the imperative need for the invention of groundbreaking therapeutic strategies. In this context, our study delves into the potentialities offered by Anticancer Peptides (ACPs), an emergent class of therapeutics that function by selectively intercalating into and perturbing the membranes of neoplastic cells. Operating under the conjecture that their inherent characteristics considerably modulate the anticancer efficacy of ACPs, we conducted a statistical examination of over 60 ACP sequences and cytotoxicity profiles. We applied multiple regression models to pinpoint the attributes wielding the most predictive power. Our investigation revealed that the peptides' net charge and the isoelectric point emerged as the most consequential parameters. Our research endeavors to craft a predictive model as a preliminary screening instrument for pinpointing prospective peptides with high therapeutic potential for breast cancer treatment. This tool could potentially economize time and resources by circumventing the investigation of peptides that, due to their intrinsic properties, have a low likelihood of demonstrating efficacious results. Ultimately, our research aims to contribute to the progress in breast cancer research to improve outcomes for the millions of people impacted each year.

KEYWORDS: Translation and Medical Sciences; Disease Treatment and Therapies; Breast Cancer; Regression Modeling.

Introduction

Female breast cancer is now the most commonly diagnosed cancer globally and the second leading cause of death from cancer in women.[1] In the United States, about 1 in 8 women develop invasive breast cancer over their lifetime, and in 2023, 297,790 new cases and 43,700 deaths due to breast cancer are expected to occur.[2]

Genetic mutations, radiation exposure, contact with carcinogens, viral infections, and lifestyle choices like smoking or binge drinking are a few of the many reasons normal cells undergo a malignant transformation into cancer cells.[3] Cancer is categorized as invasive or non-invasive based on whether it has spread from its original location to nearby tissues. Breast cancer cases are most commonly invasive, with one such class of invasive ductal carcinoma (IDC) accounting for 80% of diagnosed cases of breast cancer, followed by invasive lobular carcinoma accounting for 10-15% of cases.[4] The Tumor, Nodes, and Metastasis (TNM) system describes the stages of breast cancer from 0 to IV: Stage 0 refers to non-invasive breast cancer, in which abnormal cells have not spread beyond the milk ducts or lobules of the breast, whereas stage IV refers to metastatic breast cancer, in which the cancer has spread to other parts of the body.[5] The prognostic indicators for individuals diagnosed with stage IV breast cancer suggest a five-year survival rate of 22 percent—a percentage that increases with the stages, with a median survival duration of three years.[6]

Breast cancer is a complex disease with multiple subtypes and is often characterized based on the presence of particular molecular markers. These subtypes are crucial for deciding the therapy options and therapeutic goals. For example, endocrine therapy tamoxifen is one such treatment option for women with hormone receptor-positive tumors due to its demonstrated effectiveness and favorable safety profile.[7] Chemotherapy is commonly paired with ERBB2-targeted therapy for patients with ERBB2-positive tumors and is the only treatment given to patients with triple-negative tumors.[8] While chemotherapeutic resistance is frequently cited as a cause of treatment failure, the use of small molecule inhibitors that specifically target intracellular biochemical pathways in combinatorial therapies has been the primary strategy employed to prevent the emergence of resistant cell subpopulations. Nonetheless, current treatments induce numerous negative side effects such as nausea, vomiting, diarrhea, hair loss, soreness, and more. Therefore, new treatments are required to improve healthcare quality of life and overall treatment outcomes.

Clinical interest has been rapidly increasing in incorporating tumor lytic agents into combinatorial therapies, owing to their unique ability to rapidly kill cancer cells through physical mechanisms not present in conventional drugs.[9] Therefore, anticancer peptides (ACPs) have become a subject of growing interest as potential therapeutics for use in the era of drug resistance. This class of potential therapeutics acts by selectively intercalating into and disrupting the membranes of cancer cells. It has been shown that ACPs can act as adjuvants and improve the therapeutic outcomes of other agents.[10] At present, attempts are underway to invest in creating novel peptides that possess anticancer properties.[11] Current research endeavors are focused on developing novel peptides with potent anticancer properties. Amidst this burgeoning research landscape, our study is designed to establish a foundational understanding of the prediction and design of future ACPs.

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In this study, we hypothesize that their inherent properties significantly influence the anticancer activity of ACPs against breast cancer. We predict that some properties of these peptides will have a more substantial effect on their anticancer activity than others, indicating that certain characteristics could be more predictive of anticancer efficacy. We aim to identify the most predictive attributes through systematic statistical analysis of these key properties across different ACPs with their observed anticancer activity. Building on these insights, the comprehensive database of ACPs will be utilized to develop a predictive model. This model will be designed to serve as an initial screening tool, pinpointing those peptides with a high potential for killing breast cancer cells based on the analysis of their structural properties. Therefore, this model will potentially streamline identifying the most promising ACP candidates by efficiently suggesting peptides that warrant further rigorous experimental testing. Meanwhile, peptides with predicted lower anticancer potency can be deprioritized or removed from further consideration. This can be a valuable tool to ensure that no resources are wasted on peptides that are likely devoid of anticancer properties.

Therefore, this research will deepen our understanding of how the properties of ACPs influence their anticancer activity and may provide valuable insights into the rational design of more effective and less toxic ACP-based therapeutics for breast cancer. Furthermore, it will yield a statistical model that, pending experimental validation, could predict the anticancer activity of peptides based on their structural properties to act as an initial screening tool for the testing of effective ACPs against breast cancer.

A Brief History of ACPs and Their Evolution from AMPs:

In 1981, Cecropin, the first antimicrobial peptide (AMP) was discovered in silk moths (*Hyalophora cecropia*) and injected with bacteria.[12] Another landmark study in 1987 isolated and characterized AMPs from the African clawed frog, *Xenopus laevis*, for their role in immune defense known as magainins.[13] Thousands of AMPs have since been discovered in almost all living organisms, including plants, bacteria, fungi, and animals. Over the last decade, the use of AMPs for treating diseases has increased due to the issue of pathogenic organisms becoming resistant to traditional antibiotics.[14]

A comprehensive understanding of the role of AMPs is crucial in identifying novel, non-resistant therapies for infectious and non-infectious disorders. AMPs exhibit low antigenicity, are critical to the innate immune system's defensive mechanism, and are ubiquitous among eukaryotic organisms.[15,16] Moreover, recent research has revealed that AMPs possess anticancer properties and have thus been dubbed anticancer peptides (ACPs). The promising potential of ACPs in cancer therapy stems from their short interaction time (which mitigates the likelihood of resistance), low toxicity, specificity, good solubility, and excellent tumor penetration.[17-19] Therefore, ACPs represent an exciting avenue for developing effective cancer therapeutics.

Defining ACPs: Structure and Properties:

Small in size, ACP peptides typically comprise 5 to 50 amino acid residues. These peptides are cationic and highly hydrophobic, with a positive net charge. ACPs can interact with the anionic components of cancer cell membranes due to their charge, allowing them to target and destroy cancer cells selectively. ACPs have also been shown to promote cell lysis that results in necrosis, disrupt mitochondria, trigger apoptosis, boost the host's immune system, and inhibit tumor angiogenesis.[20] Structurally, ACPs are classified into four primary categories, namely linear, cyclic, helical, and disulfide-rich, based on their secondary structure, as shown in Figure 1.[21]

(a) Helical peptides: Hydrogen bonds and hydrophobic interactions let helical ACPs maintain their -helical structure. These peptides frequently have different cationic and hydrophobic regions that let them interact with membranes and cause cytotoxicity.[22]

(b) Cyclic peptides: Cyclic ACPs create disulfide bridges or covalent bonds between the N- and C-termini. The cyclic structure strengthens their anticancer potential, which gives higher stability and resistance to proteolytic breakdown.

(c) Linear peptides: Linear ACPs are characterized by their short length, high concentration of cationic and hydrophobic residues, and absence of particular secondary structure. These peptides often damage the cancer cell membrane through interactions with hydrophobic and electrostatic molecules.

(d) Disulfide Rich ACPs: These peptides have a pleated conformation stabilized by hydrogen bonds, with alternating hydrophobic and hydrophilic residues. Their beta-sheet structure enhances stability and proteolytic resistance, allowing them to target cancer cells based on specific amino acid motifs effectively.

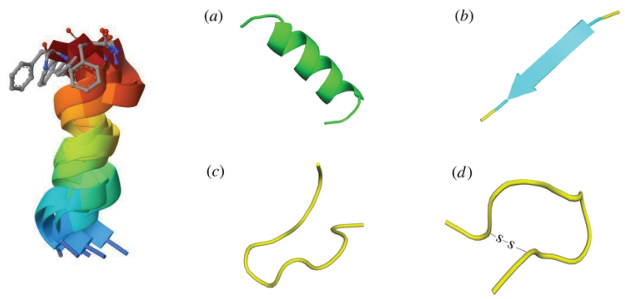

Figure 1: The 4 categories of anticancer peptides (a) Helical (b) Cyclic (c) Linear (d) Disulfide-rich (Reproduced under Open Access from Xie *et al.*, 2020)[23].

The presence of certain amino acids and their derivatives in peptides translates to specific properties. Glycine, lysine, and leucine are the main amino acid residues in peptides with anticancer properties. For instance, peptides high in the hydrophobic and positively charged amino acids lysine and arginine, which can interact with membranes using a snorkeling mechanism, may selectively target the anionic membranes found in cancer cells, causing a disruption of the integrity of the cell membrane, and possibly inducing cancer cell toxicity. In addition, histidine-containing peptides may be lethal to cancer cells by increasing the permeability of cell membranes in acidic environments since histidine is protonated in these situations. Aspartic and glutamic acid residues may also have an anti-proliferative effect on tumor cells, and cysteine-rich do-

mains on some cell surface receptors may assist in stabilizing and sustaining extracellular motifs or domain structures. Phenylalanine, a strongly hydrophobic amino acid, is commonly found in primary tumors and may function as a protective amino acid; peptides containing phenylalanine may increase the binding affinity for targeting the cancer cell membrane.[24] All of these results suggested that to create further secondary structures that impact cancer cells, ACPs should have cationic and hydrophobic amino acid residues. Refer to Table 1.

Table 1: Role of amino acid residues on ACP effects in cancer cells. This table is reproduced from Table I from Chianjong *et al.*, 2020.[25]

Amino Acid Residue	Amino Acid Properties	Action on Cancer Cells
Charged residues on ACPs		
Lysine, Arginine	Positively charged (basic amino acids)	They compromise the integrity of the cancer cell membrane and facilitate their entry into cells, destroying cancer cells.
Histidine	Polar, hydrophilic	Under acidic conditions, they can increase cell membrane permeability, resulting in cytotoxic effects on cancer cells
Glutamic acid, Aspartic acid	Negatively charged (acidic amino acids), polar, hydrophilic	They may inhibit the growth of cancer cells by suppressing cell proliferation
Effect on cancer cell structure		
Cysteine	Polar, non-charged	They interact with various cell surface receptors and help maintain the structure of extracellular motifs/domains.
Proline	Non-polar, aliphatic residues	They facilitate interactions with the cell membrane and provide conformational flexibility, potentially enhancing the cytotoxic activity against cancer cells.
Glycine	Aromatic	They participate in cell membrane interactions and provide conformational flexibility.
Phenylalanine	Aromatic	They may increase the binding affinity for target cancer cell membranes, offering protective effects to primary tumors while potentially augmenting cytotoxic activity.

ACP Mechanisms: How Do They Kill Cancer Cells?:

Cationic amphipathic peptides exhibit a remarkable ability to distinguish between benign and malignant cells by interacting selectively with negatively charged membrane constituents such as phosphatidylserine (PS), sialic acid, or heparan sulfate, which display differences between cancerous and non-cancerous cells. Additionally, an elevated number of microvilli on cancerous cells enhances their susceptibility to ACPs by increasing their surface area.[26] Likewise, it has been shown that peptides used for host defense selectively target variations between cancerous and non-cancerous cell membranes, exhibit exceptional tissue penetration in tumor cells, and can access primary tumors and distant metastases. Due to the mechanism of rapid cell death via disruption of the plasma membrane, the likelihood of resistance to these peptides is reduced.[27]

First, ACPs primarily disrupt the cytoplasmic membrane of their target. They can bind to vital molecules within the cells, causing inhibition of DNA, RNA, or protein synthesis, blocking certain enzymes and cell wall synthesis, and leading to apoptosis or necrosis. These peptides often form pores or ion channels in the membrane, causing leakage of cellular contents and cell death.[28] Some anticancer peptides also inhibit membrane fusion processes needed for cancer cell growth and immune resistance.

Second, certain anticancer peptides, such as the MAPK and PI3K/AKT pathways, inhibit intracellular signaling pathways needed for cancer cell proliferation. These peptides can directly inhibit key protein kinases in these pathways or other upstream signaling molecules. Inhibition of these proliferative signaling pathways leads to cell cycle arrest and apoptosis in cancer cells.[29]

Finally, several anticancer peptides contain immunomodulatory properties that enhance the body's ability to fight tumors. These peptides may trigger the innate immune system's natural killer cells, macrophages, and dendritic cells. They may also increase these immune cells' capacity to kill cancer cells through cytotoxicity. Certain peptides can also inhibit regulatory T cells and myeloid-derived suppressor cells, reducing the immune system's antitumor response effectiveness.[30]

Modes and Mechanisms of Membrane Disruption:

The following array of models provides insights into diverse mechanisms by which membranolytic ACPs disrupt biological membranes, as illustrated in Figure 2.[31]

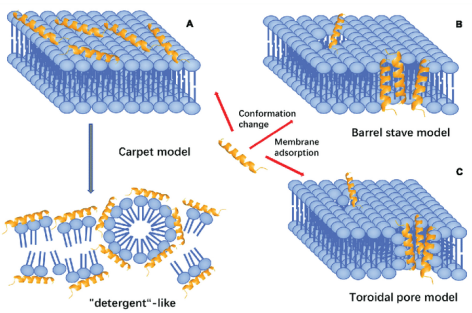

Figure 2: A diagrammatic representation of some membrane perturbation mechanisms. A) Carpet model: AMPs pile up on the surface and then break down the cell membrane, similar to how a detergent works. B) Barrel stave model: AMPs gather together and push into the cell membrane's double layer as a group. They align themselves with the phospholipids and create a channel. C) Toroidal pore model: AMPs stack up and embed themselves upright into the cell membrane before bending to form a ring-shaped hole. (Reproduced under Open Access from Huan *et al.*, 2020)

1. *Barrel-Stave Pore Formation:* Peptides are inserted perpendicularly into the lipid bilayer in the Barrel-stave model. They form a "barrel" composed of peptide "staves" surrounded by lipid "hoops". This creates aqueous channels across the membrane, disrupting its integrity by permitting the transport of ions and small molecules.
2. *Toroidal Pore Formation:* In contrast to the Barrel-stave model, the Toroidal pore formation model suggests that membrane-disrupting molecules simultaneously interact with lipid head groups and bend the lipid monolayers to form a continuous pore of lipid and peptide. This results in a transient pore, with the lipids lining the pore wall in a "toroidal" or donut-shaped configuration alongside the peptides, maintaining the continuity of the water phase across the membrane.

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3. *Carpet and Detergent-like Models:* The carpet model proposes that peptides adsorb onto the membrane surface, aligning parallel to the plane of the membrane and covering it like a carpet. Upon reaching a threshold concentration, the peptides disrupt the membrane through a detergent-like mechanism, leading to micellization and consequent membrane disintegration. This model is less structure-specific and more concentration-dependent than the pore formation models.

4. *Shai–Huang–Matsuzaki Unifying Model:* This unifying model integrates elements from the aforementioned models, suggesting a sequence of events initiated by peptide binding to the membrane surface, followed by insertion and alignment within the membrane, and culminating in the formation of transient toroidal pores. This model accommodates peptide-membrane interactions' dynamic and concentration-dependent nature, offering a more holistic view of the membrane disruption process.

5. *Membrane Composition Model:* The membrane composition model emphasizes the significance of lipid composition in membrane disruption. It posits that the presence of specific lipid species, particularly those with negatively charged head groups, enhances peptide binding, insertion, and subsequent membrane disruption. This model acknowledges the complexity and diversity of biological membranes, underscoring the importance of the lipid environment in the mode of action of membrane-active molecules.

Molecular Mechanisms of Cancer Chemoresistance and Shortcomings of Existing Therapies:

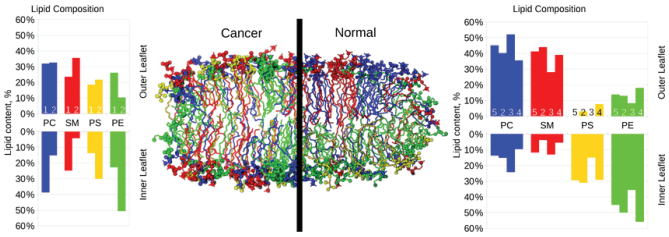

Figure 3: A simulation of comparing normal and cancerous membrane models. Different lipid species are represented by different colors: DOPC in blue, SM in red, DOPS in yellow, and DOPE in green. The histograms in the figure demonstrate the relative distribution of various lipid species within each monolayer of the membrane models. (Reproduced under Open Access from Rivel *et al.*, 2019)

The plasma membrane of cells, acting as a critical barrier and mediator in the uptake of chemotherapeutic agents like cisplatin, exhibits distinct differences in lipid composition between non-malignant and cancerous cells. In normal cells, the plasma membrane's asymmetry is characterized by a specific organization where the outer monolayer is enriched with SM (depicted in red) and DOPC (depicted in blue). In contrast, the inner monolayer predominantly contains DOPE (depicted in green) and DOPS (depicted in yellow), see Figure 3.[32] Enzymes actively maintain this asymmetrical distribution and are essential for various functions, including cell signaling and apoptosis. In stark contrast, cancerous cells tend to exhibit a loss of this lipid asymmetry, which is particularly evident in the outer monolayer's aberrant accumulation of DOPS and DOPE. This alteration not only signifies a hallmark of cancer cells but also has been implicated in the reduced permeability to cisplatin, thereby contributing to the chemoresistance observed in malignant cells.

Furthermore, the increased cholesterol content in cancer cell membranes, which also decreases membrane permeability, adds another layer of complexity to the drug resistance mechanism. The stiffening of the membrane due to cholesterol's 'condensing effect' limits the passive diffusion of small molecules like cisplatin, thus requiring alternative therapeutic approaches that can bypass or exploit these lipid-mediated barriers. The diminished permeability of cancer cell membranes necessitates the exploration of alternative therapeutic agents capable of circumventing these barriers. This further highlights the need for novel drug development of ACPs to be an innovative direction in cancer treatment that directly addresses the shortcomings of existing therapies. By targeting the altered lipid composition of cancer cell membranes, these peptides can provide a more targeted and effective approach to cancer therapy, providing better outcomes for patients facing the multifaceted challenges of chemoresistance.

Drawbacks of ACPs for Therapeutics:

As described earlier, ACPs have become the focus of research by different groups, mainly due to their ability to selectively inhibit the growth of tumor cells while maintaining resistance prevention. Many of the millions of synthetic and thousands of natural peptides created by rational design have anticancer properties. Still, only a small number have been investigated, and an even smaller number are participating in clinical trials. This is mainly because creating these peptides as pharmaceutical medications presents several difficulties, such as greater synthesis costs than organic antibiotic small molecules.[33] Moreover, the challenges in applying these peptides to therapy are further increased by the negative effects observed in some peptides, such as their high toxicity to healthy mammalian cells and low ability to modify the immune response. Therefore, it is important to separate their antimicrobial/antitumor activity from toxicity to mammalian cells to be commercially viable. This can be achieved by increasing their antimicrobial activity, decreasing their haemolytic activity, or both.

Moreover, to make the peptides more applicable for drug use, addressing their vulnerability to proteolysis is important, which limits their oral bioavailability. Although oral administration is a preferred mode of drug delivery, peptides are usually administered through traditional routes such as intramuscular or intravenous injection due to their susceptibility to proteases and poor intestinal membrane penetration. Determining the time of circulation, essential for a drug to be efficient, is also challenging. Various solutions have been suggested for this issue, including using drug carriers like bacteriophages and cell-penetrating peptides to conjugate the peptide. Covering or linking peptides with polymers, such as polyethylene glycol (PEG), can enhance the duration of circulation and pharmacokinetics/dynamics, regardless of the polymer used, by enabling better circulation and improving their infiltration into the targeted cancer cells.[34] Advantages and disadvantages of ACPs are summarized in Figure 4.

Figure 4: Strengths and Weaknesses of Peptide Therapy.

Methods

Literature Search for Breast Cancer Peptides:

A search for potential peptide candidates for breast cancer treatment was conducted using two primary resources: the Database of Antimicrobial Activity and Structure of Peptides (DBAASP) and a research paper, "Bioactive Cationic Peptides as Potential Agents for Breast Cancer Treatment" by Marcela Manrique-Moreno.[35]

The search parameters in DBAASP were kept in default settings except for the "Target Group" and "Target Species" filters. For "Target Group," the "Cancer" category was selected. Within the "Target Species" filter, all tags pertinent to breast cancer, including "human breast adenocarcinoma" and "human breast carcinoma," were selected. This yielded 838 entries, from which 24 peptides were randomly chosen for further analysis.

All peptide properties were recorded in addition to the IC50 values of these peptides, which indicate their potency in inhibiting cancer cell growth in their respective cancer cell lines.

Simultaneously, the research paper provided another set of 37 peptides with potential anticancer activity against breast cancer. However, while the research paper provided valuable information on the peptide sequences and anticancer activity, it lacked specific details about their properties. To bridge this gap, the sequences of these peptides were converted into FASTA format. Then, using the "Tools" section in the DBAASP database, each sequence was input to generate a detailed profile of its properties. The Kyte and Doolittle hydrophobicity scale was used to derive the peptide's properties.

Therefore, this dual approach of using a well-established peptide database and peer-reviewed scientific literature enabled a thorough analysis of potential peptide candidates for breast cancer treatment.

Explanation of Peptide Properties:

Below are brief explanations of the commonly recorded properties of peptides. By analyzing these properties through structural activity relationships, we aim to discern which ones most significantly affect the IC50. This quantitative measure indicates how much of a peptide is needed to inhibit a biological process by half, thereby guiding more effective peptide design for breast cancer treatment.

Table 2: Explanation of peptide physical properties.

Peptide Property	Description
Angle Subtended by the Hydrophobic Residue	This refers to the angle formed by the hydrophobic residues in a peptide's structure. This angle can influence the peptide's interaction with hydrophobic entities like cell membranes.
Hydrophobic Moment	This measures the peptide's amphipathicity, representing the distribution of hydrophobic residues in a peptide. It can influence the ability of a peptide to interact with and penetrate lipid bilayers.
Normalized Hydrophobicity	This is the hydrophobicity value of a peptide adjusted for its length. Higher values can enhance interactions with lipid bilayers and potentially influence peptide-membrane interactions.
Net Charge	This is the sum of positive and negative charges on a peptide, affecting its overall charge. It can influence the peptide's interaction with negatively charged cell membranes and solubility.
Isoelectric Point	This is the pH at which the peptide has no net charge. It can influence peptide solubility and interactions with other molecules and change the peptide's behavior under different pH conditions.
Penetration Depth	This refers to how deeply a peptide can be inserted into a lipid bilayer. This property can affect the peptide's ability to disrupt the membrane or interact with intracellular targets.
Tilt Angle	This refers to the angle at which a peptide is inserted into the lipid bilayer. This can influence the degree of disruption of the membrane.
Disordered Conformation Propensity	This measures a peptide's tendency not to form a structured 3D shape. Peptides with a high propensity for disordered conformation are flexible and can adapt to different environments or targets.
Linear Moment	This is a measure of the distribution of mass in a peptide. It can influence the peptide's rotational behavior and interactions with other molecules.
Propensity to *In Vitro* Aggregation	This measures a peptide's tendency to aggregate in vitro, influencing its activity, half-life, and potential to cause unwanted side effects.
Amphiphilicity Index	This measures the extent to which a peptide has separate hydrophobic and hydrophilic faces. This property can influence peptide-membrane interactions and the peptide's ability to penetrate the membrane.
Propensity to PPII Coil	This refers to a peptide's tendency to form a polyproline type II (PPII) helix, a secondary structure often found in proteins and peptides. This structure can influence a peptide's stability and interactions with other molecules.

Graphical Correlation Analysis of Peptide Properties:

To provide tangible examples and data that illustrate the interaction and influence of properties, and to supplement the secondary aim of our paper, which is to furnish background information about ACPs, we have done a graphical analysis of property correlation.

In this section, we analyzed the correlation between normalized hydrophobicity and four distinct parameters (chosen for its shown correlation): [A] disordered conformation propensity, [B] penetration depth, [C] amphiphilicity index, and [D] propensity to PPII. Using the value of R^2, also known as the coefficient of determination, we can use a statistical measure in the regression analysis to quantify the goodness of fit of a model. It ranges from 0 to 1 and describes what proportion of the variance in the dependent variable can be accounted for by the independent variable. An R^2 value closer to 1 signifies that a higher percentage of the data fits the regression model, indicating a strong correlation between the two variables under consideration.

Multiple Regression Model:

Constructing a cogent regression model to predict the target activity of peptides, given their properties, is a layered process. The first stage of this process was to assemble a comprehensive peptide dataset on ACPs active against breast cancer. 66 peptides were sourced from the DPSAAP database and supplemented by peptides from the research paper Bioactive Cationic Peptides as Potential Agents for Breast Cancer.[36] The peptide selection aimed for randomness to ensure a broad spectrum of property values contributing to potency against MCF-7. The next stage involved conducting regression analysis to identify which specific property significantly impacts the peptide's target activity against MCF-7. To refine the model,

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peptides with a target activity greater than 100 μM were excluded, signifying a low effect on breast cancer.

Activity Prediction Equation:

A key aspect of this study was the development of an equation (Figure 5) to predict the activity of anticancer peptides against breast cancer. This was achieved by leveraging the coefficients derived from the regression analysis.

In formulating the equation, we copied the coefficients corresponding to each property and the y-intercept into a new datasheet. Subsequently, we took the property values of each peptide and inserted them into the same sheet. This led to the creation of the following equation:

$$\sum_{r=1}^{n}(ar \times xr) + b$$

Figure 5: Coefficients as "a", Property Values as "x", and the y-intercept as "b". If we have n properties.

This equation essentially represents a linear combination of the properties of the peptide, weighted by their respective coefficients obtained from the regression analysis, with the y-intercept as an additive constant. The equation was used to generate a predicted activity for each peptide. The real target activity of each peptide, obtained through experimental data from various research papers, was then compared against the expected activity.

To evaluate the quality of the prediction, we classified outcomes into two categories: “near-perfect prediction” and “good prediction”. A “near-perfect prediction" was characterized by a maximum difference of 5μm between the predicted and real activity. In contrast, a “good prediction” was described by the difference falling within the range of 5-20μm.

Test of Equation’s Prediction Accuracy:

After successfully validating the predictive equation with a set of known peptides, the next logical step was to test its applicability on a fresh set of peptides. For this task, we randomly selected 16 additional peptides from the DBAASP database, which have known activities against breast cancer, and inputted their properties in the equation.

Test of Equation’s Prediction Accuracy Against Non-Anticancer Peptides:

To test the specificity of our model, we chose to trial it against a different family of antifungal peptides. Eleven of these were sourced from the DBAASP database.

Test of Equation’s Prediction Accuracy Against Non Breast Cancer Peptides:

To validate our prediction model against non-breast cancer peptides, we searched for ovarian and bladder cancer-targeting peptides from the DBAASP database. We randomly selected 11 peptides.

Result and Discussion

Graphical Correlation Analysis of Peptide Properties:

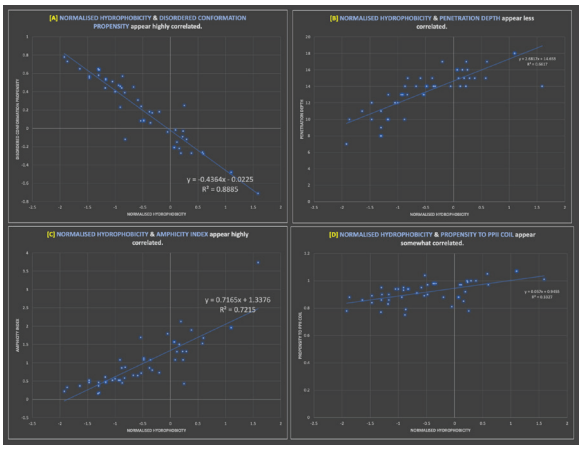

Figure 6: Correlation between Peptide Properties; Normalised Hydrophobicity and [A] Disordered conformation propensity [B] Penetration Depth [C] Amphicity Index [D] Propensity to PPII coil.

This section observed several correlations concerning the peptide's normalized hydrophobicity and other characteristics.

In Figure 6 [A], a high correlation was found between normalized hydrophobicity and disordered conformation propensity, with an R^2 value of 0.8885. In Figure 6 [B], there was a significant correlation between normalized hydrophobicity and penetration depth, represented by an R^2 value of 0.6617. In Figure 6 [C], the relationship between normalized hydrophobicity and the amphiphilicity index also demonstrated a notable correlation, with an R^2 value of 0.7215. Figure 6 [D] shows the weakest correlation was observed between normalized hydrophobicity and the propensity to PPII, with an R^2 value of 0.3327.

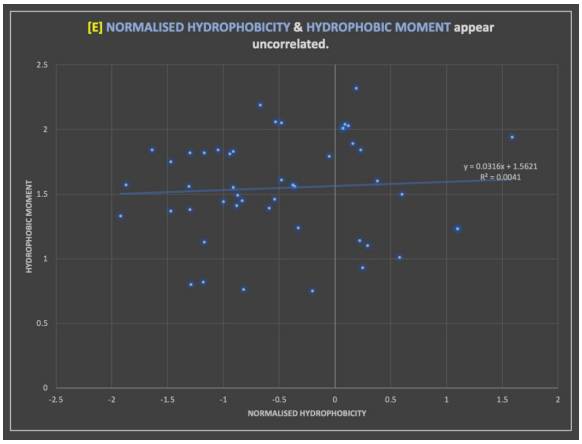

Figure 7: Correlation between Peptide Properties, Normalised Hydrophobicity, and Hydrophobic Moment.

In Figure 7 [E], a surprisingly low correlation was found between normalized hydrophobicity and the hydrophobic moment, as demonstrated by an R^2 value of 0.0041.

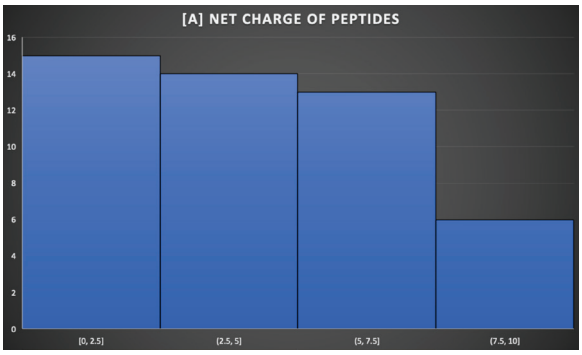

Figure 8: Histogram of the net charge of anticancer peptides used in the structure-activity relationship analysis. The results reveal a right-skewed distribution favoring the mean net charge between 2.5-5.

In Figure 8. It can be noted that the range of the peptide charge primarily falls within the 0-10+ spectrum. Peptides with a charge state of 0, i.e., neutral entities were observed; peptides carrying a low positive charge, ranging from 1-3+, were also noted; peptides with moderately positive charge states, falling within the 4-6+ bracket, were present; peptides with high positive charge states, from 7-10+, were also part of the database.

Multiple Regression Model:

Table 3: Outcomes of different regression models after refinement. Properties with notable P-values are bolded.

Regression Statistics		
Multiple R		0.749693
R^2		0.562040
Property	**Coefficients**	**P-value**
Angle Subtended By The Hydrophobic Residue	-0.214952187	0.385120935
Hydrophobic Moment	-1.40564067	0.954959824
Normalised Hydrophobicity	**28.25356483**	**0.349259372**
Net Charge	**-17.74324938**	**0.003113282**
Isoelectric Point	**17.50931973**	**0.000649118**
Penetration Depth	0.432978832	0.921081587
Tilt Angle	-0.151212459	0.545361822
Disordered Conformation Propensity	-73.30077209	0.465454881
Linear Moment	-5.124737086	0.946399637
Propensity To In Vitro Aggregation	-0.006630327	0.865404954
Amphiphilicity Index	**-38.21558647**	**0.130993607**
Propensity To PPII Coil	**-113.9249251**	**0.145035337**

The initial regression model incorporating all peptides resulted in a multiple R (multiple correlation coefficient) of 0.590, indicating a moderate relationship between the dependent variable (target activity of peptides) and the set of independent variables (peptide properties). After excluding peptides with a target activity greater than 100 μM, the re-run of the regression analysis improved the multiple R to 0.7497, suggesting a stronger relationship. Net Charge and Isoelectric Point stood out with extremely low p-values of 0.003113 and 0.0006491, respectively, indicating a strong influence on peptide activity. Other properties like Amphiphilicity Index (p-value: 0.1310), Propensity to PPII Coil (p-value: 0.1450), and Normalised Hydrophobicity (p-value: 0.3493) also exhibited relative significance.

Activity Prediction Equation:

Out of the 58 peptides tested using the equation, the model could predict the activity of 26 peptides to at least a "good prediction" extent, representing an accuracy of about 44%. Nine of the peptides displayed a "near-perfect prediction."

Test of Equation's Prediction Accuracy:

After inputting these peptides' properties into the predictive equation, it achieved a 'good prediction' accuracy for 4 out of the 16 peptides. This represents an accuracy of 25%, a somewhat lower rate than observed with the initial set of peptides.

Test of Equation's Prediction Accuracy Against Non-Anticancer Peptides:

The equation failed to predict the activity of any of the 11 randomly chosen antifungal peptides.

Test of Equation's Prediction Accuracy Against Non-Breast Cancer Peptides:

The equation failed to predict the activity of any of the 11 randomly chosen ovarian and bladder cancer-targeting peptides.

Graphical Correlation Analysis of Peptide Properties:

In Figure 6 [A], the relatively high correlation (R^2 = 0.8885), means the changes in normalized hydrophobicity can account for significant variation in the disordered conformation propensity). This suggests that as the hydrophobicity of a peptide increases, its propensity to adopt a disordered conformation also increases. The disordered conformation is a state where the peptide does not adopt a well-defined 3D structure. This could result from the hydrophobic residues clustering together to minimize contact with the aqueous environment, leading to a less ordered and more flexible structure. The high correlation might also reflect the peptide's adaptability in the complex biological milieu. Disordered conformations often exhibit high structural plasticity, allowing them to interact with various molecular targets, including those involved in cancer progression. Therefore, the correlation between hydrophobicity and disordered conformation propensity could be an important factor when considering the anticancer activities of these peptides.

In Figure 6 [B], there was a significant correlation between normalized hydrophobicity and penetration depth, represented by an R^2 value of 0.6617. This may be due to how hydrophobic peptides tend to embed more deeply into the bilayer due to their affinity for the hydrophobic core of the lipid bilayer; the greater the hydrophobicity, the deeper the peptide penetrates the bilayer. Many of these peptides exert their anticancer effects by disrupting the cancer cell membrane. Therefore, a deeper penetration might allow these peptides to cause more substantial disruption to the lipid bilayer, potentially increasing their anticancer efficacy.

In Figure 6 [C], the relationship between normalized hydrophobicity and the amphiphilicity index also demonstrated a high correlation, with an R^2 value of 0.7215. The amphiphilicity index measures the peptide's ability to form amphipathic structures, which are key to their interaction with the lipid bilayer. As the hydrophobicity increases, peptides tend to form

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these structures, enhancing their interaction with the cell membrane. The amphipathic nature of these peptides allows them to align themselves at the membrane interface, with the hydrophobic face interacting with the lipid core and the hydrophilic face exposed to the aqueous environment. This unique orientation can facilitate membrane disruption and subsequently, the anticancer activity of these peptides.

In Figure 6 [D], the weakest correlation was observed between normalized hydrophobicity and the propensity to PPII, with an R^2 value of 0.3327. Despite the lower correlation between normalized hydrophobicity and propensity to PPII, an increase in hydrophobicity may still increase the propensity for PPII formation. The PPII helix is extended and flexible, potentially enhancing the peptide's ability to interact with and disrupt the cell membrane. However, the weaker correlation suggests that while hydrophobicity might influence PPII propensity, other factors could also play a significant role.

In Figure 7 [E], despite both properties being related to the hydrophobic nature of the peptide, the low correlation between normalized hydrophobicity and the hydrophobic moment may initially appear counterintuitive. One might assume that as the overall hydrophobicity of the peptide increases, its hydrophobic moment, which measures the spatial distribution of hydrophobicity within a peptide's structure, should also increase. However, our analysis suggests a different interpretation.

The key to untangling this apparent contradiction lies in understanding that these two properties, despite both being rooted in the concept of hydrophobicity, describe different aspects of a peptide's nature. Normalized hydrophobicity is a measure of the overall "oil-loving" character of a peptide. In contrast, the hydrophobic moment is more about the peptide's spatial configuration, particularly the distribution of hydrophobic residues about its 3D structure. A peptide can have a high normalized hydrophobicity if it contains many hydrophobic residues, irrespective of their arrangement. However, to have a high hydrophobic moment, these hydrophobic residues need to be arranged in a specific pattern, such as being clustered on one side of the peptide. Therefore, a highly overall hydrophobic peptide might not necessarily have a high hydrophobic moment if its hydrophobic residues are scattered around its structure rather than clustered. This low R^2 value underscores the complexity of peptide behavior and the multi-faceted nature of hydrophobicity as a property. It further highlights the importance of considering multiple parameters and their inter-relationships in the analysis of peptide activity prediction. While it might be tempting to oversimplify and assume direct relationships between seemingly related properties, this result serves as a reminder that such assumptions may not hold in the nuanced world of peptide structure and function.

For Figure 8, the peptide charge range primarily falling within the 0-10+ spectrum suggests that the peptides in the database predominantly carry no charge or a positive charge up to +10. This observation provides valuable insights into the peptides' potential behavior, interactions, and applications. Moreover, peptides carrying no net charge, i.e., with a charge state 0, are neutral entities. They maintain a balance between positive and negative charges. This neutrality could limit their interactions with charged molecules or surfaces and influence their solubility properties, causing them to differ from their charged counterparts. Peptides with a low positive charge, ranging from 1-3+, display enhanced solubility in aqueous environments. This attribute is due to the existence of positively charged amino acid residues. These low positive charges can also facilitate interactions with negatively charged entities, such as DNA or cellular membranes. Progressing to peptides with moderately positive charge states, falling within the 4-6+ bracket, we observe potential enhancements in ionization efficiency during mass spectrometry experiments. The augmentation of the charge can lead to an uptick in ion intensities, thereby boosting detection sensitivity. This range of charge states could also impact the peptide's conformation and stability. Lastly, considering peptides with high positive charge states, from 7-10+, we expect increased electrostatic repulsion among the positively charged amino acid residues. This push and pull can impact the peptide's conformation, possibly causing a shift towards more extended or unfolded structures. Such high charge states might also alter the peptide's fragmentation patterns during mass spectrometry experiments.[37] However, it's crucial to remember that these generalized observations may not always hold. The specific properties and behaviors of peptides can diverge depending on their sequence, length, and composition. External factors, including solvent conditions, pH, and other environmental variables, may also impact a peptide's charge-dependent attributes.[38]

Multiple Regression Model:

The preliminary regression model, incorporating all peptides, resulted in a multiple R of 0.590. The multiple R, or multiple correlation coefficient, indicates the strength of the relationship between the dependent variable (here, target activity of peptides) and the independent variables (peptide properties). A value close to 1 suggests a strong relationship, while a value closer to 0 implies a weak relationship. A multiple R of ~0.59 signifies a moderate relationship, leaving room for model improvement. To refine the model, we excluded peptides with a target activity greater than 100 μM, which signifies a low effect on breast cancer. After this exclusion, a re-run of the regression analysis boosted the multiple R to 0.7497, which indicates a considerably stronger relationship than before. Additionally, the study of p-values could further shed light on the significance of individual properties. P-values are used in hypothesis testing to measure the strength of evidence supporting a null hypothesis. A low p-value (<0.05) would suggest that the property significantly affects the target activity. Net Charge and Isoelectric Point stood out with extremely low p-values of 0.003113 and 0.0006491, respectively, indicating a strong influence on peptide activity. Furthermore, other properties also exhibited relative significance, albeit to a lesser extent. These included Amphiphilicity Index (p-value: 0.1310), Propensity to PPII Coil (p-value: 0.1451), and Normalised Hydrophobicity (p-value: 0.3493). Despite their higher p-values, these parameters should not be disregarded, as they still contribute to the overall model.

However, the overall multiple regression performance deteriorated when the regression was re-run to exclude properties

with low p-values. This outcome underscores the multifaceted nature of peptide activity and the complex interplay of various peptide properties that contribute to it. While it might be tempting to simplify the model by focusing on one or two properties with the most significant p-values, my observations suggest that such an oversimplification could lead to less accurate predictions. This is because peptides are intricate biological molecules with a wide array of properties, each contributing in some way to their overall behavior and activity. Even properties with relatively higher p-values, which might be considered less significant in a statistical sense, could still have meaningful biological impacts. Moreover, the relationships between these properties and peptide activity might not be linear or straightforward. Certain combinations of properties might synergistically enhance peptide activity, while other combinations might have counteracting effects. Thus, excluding properties based on their p-values could inadvertently exclude these complex interactions from the model, leading to less accurate predictions, and this formed the decision to include all properties in the modeling of the equation to predict target activity.

Activity Prediction Equation:

A "good prediction" for 44% of peptides and a "near perfect prediction for 15% of peptides" suggests that despite its simplicity, the equation holds considerable predictive power and could be useful, especially when dealing with a large population of peptides. It should be noted that the thresholds of 5μm and 20μm for 'nearly perfect' and 'good' predictions, respectively, were chosen considering the inherent variability in experimental measurements of peptide activity. The nature of biological systems often introduces experimental error, and peptides can exhibit a wide range of activities. These thresholds attempt to accommodate that variability while maintaining a meaningful distinction between 'good' and 'near perfect' predictions.

The utility of this equation is particularly apparent in cases where resources for exhaustive testing of all peptides are limited. As a preliminary screening tool, the equation can effectively identify and eliminate peptides predicted to have low anticancer activity against breast cancer. Consequently, this will help save resources by reducing unnecessary testing, effectively streamlining the process of anticancer peptide selection.

Test of Equation's Prediction Accuracy:

Despite only showing accuracy on 25% of the 16 peptides tested, the focus here is not solely on the absolute prediction accuracy but also on the relative ranking of peptides by their predicted activities. Interestingly, the model consistently generated high predictive values for peptides with a high level of actual activity, i.e., above 100. This suggests that while the equation may not be able to predict the exact target activity of a peptide, it appears to be quite adept at identifying peptides with high activity levels.

This is a significant observation as it points towards the potential use of the equation as a screening tool rather than a precise predictive model. In other words, the equation could screen many peptides and identify those with low target activity values (which are potent for killing breast cancer cells). Such peptides could then be subjected to more rigorous experimental testing, while peptides with high-value predicted activities could be deprioritized or eliminated from further consideration. Therefore, this finding aligns with the reality of computational modeling in biological research. Computational models are rarely able to capture the full complexity of biological systems and, hence, are often used as preliminary screening tools rather than definitive predictors. In this context, the predictive equation developed in this study shows great promise. Furthermore, the results from this extended test suggest that the equation could be particularly useful in resource-limited settings. By efficiently screening out peptides with high target activity values, the equation allows for a more focused allocation of resources to peptides with promising anticancer potential. This type of preliminary screening can save significant resources, enabling researchers to focus their efforts on the most promising candidates. It is important to emphasize that the findings from this study are preliminary, and further refinement of the equation is warranted. Nevertheless, the initial results are encouraging and suggest that with further validation and optimization, the predictive equation could become a valuable tool in the search for effective peptides.

Test of Equation's Prediction Accuracy Against Non-Anticancer Peptides:

The idea behind the testing equation in predicting the target activity of non-breast cancer peptides was to challenge our model with a distinct set of peptides and observe whether it could maintain its predictive accuracy. If the model could accurately predict the activity of these antifungal peptides, it would suggest a lack of specificity. However, when we tested the model against a randomly selected strain, *Escherichia coli* ATCC 25726, it failed to predict the activity of any of the 11 randomly chosen antifungal peptides. This result indicates the model's fine-tuning to breast cancer peptides' unique properties and characteristics, enhancing its effectiveness and specificity as a predictive tool for anticancer peptides.

Test of Equation's Prediction Accuracy Against Non-Breast Cancer Peptides:

Our investigation's primary objective was determining our model's predictive capacity on peptides derived from non-breast cancer sources. The aim was to challenge our model with a unique set of peptides and determine if it maintained its predictive accuracy. If our model could accurately predict the activity of these distinct peptides, it would indicate a lack of specificity in its predictive capabilities.

To assess this, we selected peptides from ovarian and bladder cancers, two types of non-breast cancer. Our model predicted the activity of 11 peptides randomly chosen from this set. However, in this test, our model could not accurately predict the activity of any of the selected peptides.

This result suggests that our model is uniquely attuned to the distinct characteristics of breast cancer peptides. Its inability to predict the activity of peptides from ovarian and bladder cancers underscores its specificity and precision for breast cancer peptides.

The question arises as to why these peptides are specific to breast cancer cells. One potential explanation could be the unique lipid profiles of breast cancer cells, particularly those of

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HER2+ breast cancers. It's hypothesized that specific types of lipids may either facilitate or obstruct the peptide's capacity to penetrate the cell membrane and exert cytotoxic effects.

ACPs, due to their amphipathic nature, interact with the lipid bilayer, leading to cell membrane disruption and subsequent cell death. However, the efficacy of this process could be impacted by the specific lipid composition of the cell membrane. For instance, lipid rafts, which are more rigid due to their high cholesterol concentration and sphingolipids, could potentially restrict the penetration of ACP.

Hence, the unique lipid composition, particularly in HER2+ breast cancer cells, might play a critical role in determining the efficacy of ACP. As demonstrated by our model, the specificity of ACPs for breast cancer peptides should be considered for future therapeutic developments and targeted and potentially more effective treatment strategies.

Review of Model Existing Prediction Models (DBSAAP):

Table 4: Existing models for the Prediction of AMPs are found in the DBSNP database.

Model Name	Prediction For	Paper Reference
Prediction of Linear Cationic Antimicrobial Peptides Based on Characteristics Responsible for Their Interaction with the Membranes	Cationic Antimicrobial Peptides	39
Strain-specific antibacterial peptide prediction based on machine learning, peptide sequences, and bacterial genomes	*Escherichia coli* *Pseudomonas aeruginosa* *Klebsiella pneumoniae* *Staphylococcus aureus* *Bacillus Subtilis* *Salmonella typhimurium* *Acinetobacter baumannii* *Enterococcus faecalis*	40
Antifungal peptide prediction based on strain-specific AMP prediction algorithm described in Briefings in Bioinformatics	*Candida albicans* *Saccharomyces cerevisiae*	41
Prediction of peptides inhibited virus entry.	DENV-1 DENV-2 HIV-1 Japanese encephalitis virus (JEV) MERS-CoV SARS-CoV SARS-CoV-2 West Nile virus Zika virus Hepatitis C virus HSV-1 Vesicular Stomatitis Virus	
Comparative analysis of machine learning algorithms on the microbial strain-specific AMP prediction	Hemolytic activity of peptides	
Access the models: https://dbaasp.org/tools?page=linear-amp-prediction		

The study by Vishnepolsky and Pirtskhalava proposes a similar approach to my model—a model centered around the physicochemical properties of peptides. Interestingly, unlike traditional methods, the authors don't pool all AMPs together. They argue that different classes of AMPs have unique mechanisms of action, structure, and membrane interaction modes. This suggests that each class may require a specialized prediction approach, a hypothesis that, in my opinion, has palpable merit. The model they present is anchored on three main descriptors: hydrophobic moment, charge density, and location of the peptide along the membranes. The hydrophobic moment mirrors the peptide's ability to integrate into the membrane. At the same time, the charge density and location provide clues to the peptide's potential to interact with the anionic bacterial membrane. Performance-wise, their model showed impressive accuracy, matching, and even surpassing more complex machine learning tools for the training and test sets. This suggests that their more straightforward, property-based approach has substantial potential. Yet, I can't help but wonder about the generalizability of this model. The authors concentrated on LCAPs, which they argue make up the largest class of AMPs. What about the other AMP classes? Could analogous property-based models be devised for them? With the authors advocating for class-specific models, it would have been interesting to explore this avenue further.

In conclusion, this study reaffirms the value of a simplistic approach in predicting the antimicrobial activity of LCAPs. It echoes my work on structure-activity analysis of breast cancer peptides. Instead of complex discriminative methods, the author focuses on peptides' physicochemical properties and injects fresh air into the field. Their work suggests that returning to the basics can sometimes yield the most valuable insights.

Limitations of the Structural Activity Relationship Analysis:

While the structural activity relationship analysis has yielded valuable insights into the properties that influence peptide activity against breast cancer cells, it's important to note its limitations. The current model only considers a limited set of physicochemical properties and may overlook other potentially influential factors such as peptide length, sequence composition, and the secondary structure of the peptides. The model also does not account for the cellular context in which these peptides operate, including other biomolecules and the characteristics of the breast cancer cell lines. The complex and dynamic nature of biological systems can influence how these peptides interact with their targets, and this complexity cannot be fully captured by a model that only considers physicochemical properties. Moreover, the presence of outliers in the data suggests that there may be peptides with unique structural features or behaviors that do not conform to the general trends observed. These outliers may represent a new class of peptides whose properties and activities are not accurately captured by the current model. Lastly, the model's predictive power, as indicated by the R-squared values, is relatively low for some properties, suggesting that these properties alone are insufficient to explain the variation in IC50 activity. Thus, it is crucial to consider an integrative approach that considers multiple properties and their interplay to predict peptide activity accurately.

Despite the limitations above, this study indicated that future work could focus on incorporating other potentially influential factors into the model to improve the model's predictive accuracy and provide a more comprehensive understanding of the structural activity relationships of breast cancer peptides. Additionally, an interesting future direction is the exploration of the outliers observed in the data. These peptides could represent a new class with their unique structural features and behaviors. Detailed investigation of these outliers could uncover new structural activity relationships and expand our understanding of peptide interactions with breast cancer cells. Moreover, an in-depth exploration of the cellular context in which these peptides operate, including the presence of other biomolecules and the characteristics of the breast cancer cell lines, could provide valuable insights. Such research could reveal how the complex interplay between peptides and their biological environment influences their activity, contributing to a more nuanced understanding of peptide-based therapeutics.

In sum, while this study has made strides in understanding the structural activity relationships of breast cancer peptides, there remains a vast landscape of uncharted territory. The results presented here underscore the potential of this line of research and pave the way for exciting future investigations.

Conclusion

The study reveals that our predictive model can be reliable in identifying promising candidates for peptide-based breast cancer research. The model uses structural activity relationships to screen potential breast cancer peptides from a larger pool effectively. Although it doesn't provide exact activity values, it's efficient at spotlighting peptides with substantial potential for further testing. This efficiency can help reduce research costs and time by eliminating fewer promising candidates early in the process. Nevertheless, it is essential to note that this model is not a substitute for laboratory testing; in-depth laboratory investigations remain crucial to validate selected peptides' potential and subsequent development into effective therapeutic agents.

The model's performance was less satisfactory when predicting the activity of general antimicrobial peptides. However, this outcome underscores the model's specificity to its trained domain—breast cancer peptides—rather than a limitation. This specificity suggests that the model is better tuned to the unique properties of breast cancer peptides and less effective with peptides outside its trained domain. Our model offers an advantage over existing Vishnepolsky predictions by predicting peptides beyond Vishnepolsky *et al.* selected categories. This ability to predict a broader range of peptides underscores the model's potential in breast-cancer-targeted peptide drug discovery.

Our study unveils the potential of predictive equations in peptide-based breast cancer research, providing a promising pathway toward more efficient, targeted, and cost-effective therapeutic discovery. Future work will aim to refine our equation and explore its applicability to other peptide classes, broadening its utility in peptide drug discovery.

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