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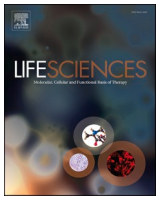

Phage display screening in breast cancer: From peptide discovery to clinical applications

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ABSTRACT

Breast cancer is known as the most common type of cancer found in women and a leading cause of cancer death in women, with the global incidence only increasing. Breast cancer in Malaysia is also unfortunately the most prevalent in Malaysian women. Many treatment options are available for breast cancer, but there is increasing resistance developed against treatment and increased recurrence risk, emphasizing the need for new treatment options. This review will focus on the applications of phage display screening in the context of breast cancer. Phage display screening can facilitate the drug discovery process by providing rapid screening and isolation of peptides that bind to targets of interest with high specificity. Peptides derived from phage display target various types of proteins involved in breast cancer, including HER2, C5AR1, p53 and PRDM14, either for therapeutic or diagnostic purposes. Different approaches were employed as well to produce potential peptides using radiolabelling and conjugation techniques. Promising results were reported for *in vitro* and *in vivo* studies utilizing peptides derived from phage display screening. Further optimization of the protocols and factors to consider are required to mitigate the challenges involved with phage display screening of peptides for breast cancer diagnosis and treatment.

1. Introduction

1.1. Overview of breast cancer (incidence, challenges)

Breast cancer has been known to be the most common cancer found in women and one of the leading causes of cancer deaths in women. Breast cancer is cancer that arises from breast tissue and are usually from the inner lining of lobules [1,2]. The global incidence of breast cancer is increasing, and it is estimated that 1 in 4 women have breast cancer, with 1 in 8 women having died due to breast cancer [3]. In Malaysia, breast cancer is the most prevalent cancer in Malaysian women [4]. Fortunately, there has been increased cases being diagnosed earlier thus improving the survival rates of breast cancer especially in younger women [2].

Breast cancer is divided into several subtypes based on the presence or absence of molecular markers for human epidermal growth factor 2 (HER2) and progesterone or estrogen receptors. There are about 70 % of patients that are HER2-negative or positive for hormone receptors (Normal like, Luminal A and Luminal B), 15 to 20 % that are positive for HER2 and about 15 % that are triple-negative (Triple Negative Breast Cancer) as the 3 standard molecular markers are absent [3,4]. The TNBC subtype can further be classified based on the gene expression into different groups such as Basal like-1, Basal like-2, Mesenchymal and Luminal Androgen [3]. As such, different subtypes will exhibit differing histopathological and clinical features as well as having differing epidemiologies, such as premenopausal and younger women having higher incidences of TNBC and HER2 positive subtypes, thus having a higher metastatic potential along with a higher relapse rate [1,5–9]. A more hectic and stressful lifestyle as well as delayed age for marriage and first child are some factors that lead to the incidence of breast cancer being higher in developed nations as compared to developing nations, where the main factors leading to high incidence and mortality from breast cancer are due to a lack of awareness, delayed diagnosis, inadequate amounts of medical facilities and improper screening programs [10–13].

Despite the worrying increase in annual incidence, there are many treatment options available for breast cancer. This includes

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radiotherapy, chemotherapy, immunotherapy, surgery and endotherapy [2,14]. However, there has been increasing resistance developed towards the treatment options mentioned as well as there being a risk of recurrence, where about 30 % of early stages of the diseases have recurrence and mostly metastasize [15]. The 5-year relative cancer-specific survival rate for metastatic breast cancer is low at 29 % as compared to 90.3 % for the 5-year relative breast cancer-specific survival rate. It is reported that the survival rate can decrease to 12 % for metastatic TNBC. Hence, it is crucial that new strategies for treatment of metastatic breast cancer, especially TNBC, be developed [15].

1.2. Role of peptide-based therapies in cancer diagnosis and treatment

Strategies to target tumors are said to be outlined as passive and active targeting, where passive targeting enhances the permeability and retention effect that result from cancer cell adaptation, thus allowing therapeutic agents to avoid targeting normal tissue but accumulate in tumor cells, reducing toxicity effects on the normal cells [16]. On the other hand, active targeting strategies use drug molecules and delivery systems such as nanoparticles to deliver antineoplastic compounds to specific or overexpressed tumor cell receptors. However, issues that arise in the therapeutic strategies are non-specific toxicity, compounds escaping from the endosome as well as challenging targeting for different cells. As such, peptides have shown promise to alleviate the challenges when attempting to transport drugs for cell internalization [16]. Peptides are polymers comprising less than 40 amino acids, thus having lower molecular weights to easily penetrate tissues than proteins [16]. Peptides tend to have an increased ability to mimic biological interactions than small drug molecules. Besides, peptides are more easily produced and modified, as well as having a lower risk of drug resistance as they have limited interactions with drug transporters. However, peptides have lower stability in the blood circulation as they can be degraded by proteases, thus having a shorter circulation half-life and lower bioactivity [17–19]. Peptide-based drugs may also be disadvantaged due to a reduced ability to penetrate the blood-brain barrier as well as polyfunctional properties [20,21]. New developments seeking to alleviate these issues include PEGylation, substituting amino acids, cyclization and configuring amino acid stereochemical properties.

There is increased demand for alternative cancer treatment options which drove the sales of peptide drugs, where there are about 100 peptide drug products currently being circulated in international markets ever since the introduction of insulin. It is estimated that the worth of the global market of peptide-based drugs by the year 2025 would reach close to USD 50 billion with the compound annual growth rate being 9.4 % [22–24]. Hence, phage display libraries as an example of *in vitro* screening techniques are strategies to accelerate the discovery of peptide-based drugs for therapeutic uses.

2. Phage display technology (fundamentals and applications)

Phage display is a molecular biology technique utilizing viruses with the capability to infect bacteria, also known as bacteriophages or phages, to present peptide and protein libraries [25,26]. This allows for the screening of therapeutic peptides for cancer diagnosis and treatment, investigating protein-ligand interactions or binding sites, drug discovery and modifying protein binding affinity (Fig. 1) [27–31]. Phage display allows for the ability to isolate peptides or proteins that bind to a target of interest with high specificity. This technique involves the expression and production of a large amount of a variety of proteins such as antibodies on peptides on the surfaces of filamentous phages after the insertion of DNA sequences of interest in the genome of the mentioned phage [25]. The proteins expressed will form a fusion product to a phage coat protein thus allowing for its display on the surface. Hence, this circumvents the issue of having to tediously analyze genetically engineered proteins or peptide variants as phage display libraries would be able to accommodate billions of variants, thus allowing for the ease of selection and purification of specific variants [25,26].

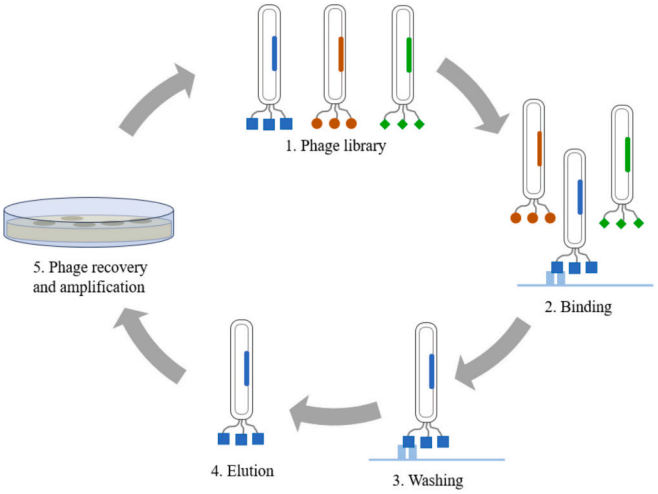

Fig. 1. The five main steps in biopanning or affinity selection in phage display.

Phages are tools with a wide variety of roles including for diagnostics and therapeutics, especially for cancer due to their ability to be engineered to target antigens specific to tumors (Fig. 2) [32]. The phages that can be used for treating cancer through recombinant technology include the T4, T7 and Lambda phages, the F, M13, fd and f1 class of phages, with the M13 filamentous phages showing a higher efficacy [32]. Such strategies require that the phages express the specific peptide as a coat protein as well as being able to disturb the complex matrix of tumor cells [32]. The filamentous M13 phage is a well-established platform and is usually used for phage display. This is because the phages are able to replicate with a higher capacity while also accommodating for the insertion of larger sizes of foreign DNA [33].

2.1. Phage display libraries selection strategies

The process of selecting phage clones that bind selectively to the target of interest is known as affinity selection or biopanning [33]. This process is required as the library will consist of billions of clones of bacteriophages where each clone would be inserted with distinct DNA to allow for the expression of different peptides on the surface, thus requiring efficient systems to select appropriate peptides for the screening process (Fig. 1). There are five main steps in biopanning, with the preparation of phage-display peptide library being done first, followed by incubation of the desired target materials such as immobilized cell surface proteins or coated antigens, with the naive library culture in microtiter plates. This is subsequently followed by removing the unbound or non-specific binders by washing so that the binders with high affinity remain. Next, bacteria are infected after eluting with acid or

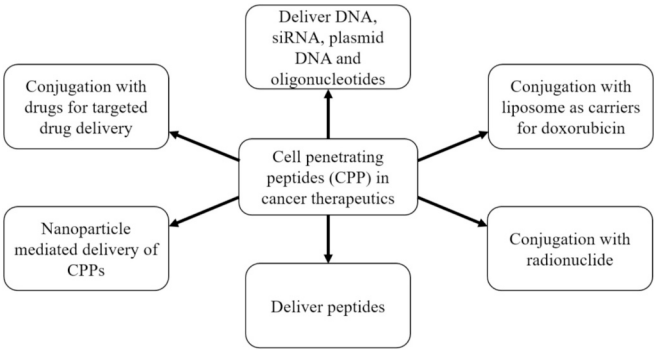

Fig. 2. Cell penetrating peptides in cancer therapeutics.

high salt solutions to allow for the recovery and amplification of the bound phages. Finally, the steps mentioned before this are repeated about three to four times to enrich the population of best binders [33]. This repetition allows for the number of phages that recognise the target to increase [34]. The individual phage clone genome encoding the displayed peptide then be sequenced to find the ligands that are specific for the target receptors. In the context of cancer therapeutics, phages can display specific targeting peptides that are able to bind to specific ligands or biomarkers, facilitating personalized treatment [34].

The screening procedures to identify peptides from the phage display peptide library are *in situ*, *in vitro* and *in vivo* selections. For peptides that target tumors, *in vitro* phage biopanning or *in vitro* whole-cell panning is used [35]. For example, Dai et al. [35] used *in vitro* phage biopanning to identify peptide AP8 to bind with acidic fibroblast growth factor found in breast cancer. This was done by infecting the phages with *E. coli* culture and the eluted phages for peptide identification. *In vitro* whole cell panning on the other hand is where peptides are identified using a single cell line or adherent cells [33].

Phage display library is a platform preferred by many to isolate specific receptor-targeting ligands due to its advantages of being able to produce a combinatorial library with a large size, hence being able to allow for more efficient identification of target-binding ligands from screening the displayed phage library as compared to conventional screening techniques which are labor intensive as individual molecules are screened at a time [33]. Phage libraries are able to be easily established, manipulated as well as cost-effective as the phage clones that display the ligands can be amplified and enriched by simply infecting a bacterial host [33]. Besides that, phage libraries are able to uncover the interactive spaces between receptors and displayed ligands without requiring knowledge about the nature of the interaction [33]. It was reported that the foreign peptides displayed also did not interfere with the ability of the phage to infect, hence allowing for the *in vitro* selection of desired antibody fragments through phage display [36].

3. Molecular targets and biomarkers in breast cancer

Breast cancer pathogenesis has been reported to be associated with two main molecular targets, estrogen receptor alpha and HER2 receptor [1]. Estrogen receptor alpha is found in about 70 % of invasive breast cancers due to its role to activate oncogenic growth pathways in breast cancer cells as a steroid hormone receptor [1]. The expression of progesterone receptors can be tracked as a marker of estrogen receptor alpha signaling due to them being highly related. As such, tumor cells expressing either estrogen receptors or progesterone receptors in at least 1 % will be categorized as HR+. Treatment for this subtype mainly relies on endocrine agents that downregulate estrogen receptor signaling. Next, HER2 is a transmembrane receptor tyrosine kinase that is part of the epidermal growth factor receptor family. HER2 is known to be associated with its upregulation in about 20 % of breast cancers as well as poor prognosis without systemic therapy [1]. Treatment for HER2+ breast cancers typically use HER2-targeted therapy using anti-HER2 antibodies and small molecule tyrosine kinase inhibitors. The leftover 15 % of breast cancers are grouped as triple-negative breast cancer as they are characterized by the lack of expression of the molecular targets. This subtype of breast cancer is reported to have a high risk of distant recurrence within the first 3 to 5 years after diagnosis [1]. Early diagnosis is crucial as Stage I breast cancers typically have high 5-year breast cancer-specific survival of at least 85 %, while Stage IV breast cancer patients with HR+ or HER2+ subtypes have median overall survival of about 5 years while those with TNBC subtype have about 1 year [1].

Other biomarkers have been identified to assist in diagnosis, prognosis, drug resistance as well as therapeutics, such as alterations in the DNA methylation pattern as it is reported that at least 90 % of breast cancer patients have methylated promoter of adenomatous polyposis coli and retinoic acid receptors-2 gene. Besides, breast cancer occurring in adolescence was implicated to be associated with hypomethylation of hyalurono glucosaminidase 2 and S100 calcium-binding protein P [37,38]. It has been recently discovered that dysregulated noncoding RNAs including microRNAs (miRNAs) and circular RNAs (circRNAs) have promising roles as noninvasive diagnostic and prognostic markers for breast cancer as well. Some noteworthy noncoding RNAs include miR-221, miR-145 and miR-21 which have shown to have diagnostic susceptibility when compared to carcinoembryonic antigen and cancer antigen 15–3 [39–41]. Further examples include upregulated hsa_circ_103110, hsa_circ_104689 and hsa_circ_104821 [42].

Phage display has shown potential in identifying potential biomarkers for breast cancer. Pavoni et al. [43] demonstrated the usage of lambda bacteriophage to display potential targets in breast cancer. Unknown proteins were found in the tumor antigen panel established from the construction of cDNA phage-displayed libraries from breast cancer cell lines, MCF-7 and MDA-MB-468 cells. Out of the eight unknown proteins identified, two were observed to often be upregulated in breast cancer. One particular unknown antigen, T7–1, was found to have good reactivity with sera of patients with tumors, thus identified as a potentially promising target. Besides these molecular targets, it is suggested that artificial intelligence can help to determine the correlation of cell cycle markers and proliferation markers for personalized treatment [44]. Genome profiling of each patient can also help to navigate molecular heterogeneity in personalized medicine [45–47].

4. Phage display screening for breast cancer diagnosis

Phage display screening technology can be used to detect biomarker molecules to facilitate disease screening, especially cancer (Table 1) [34]. Breast cancer is a prevalent malignancy with high mortality rates globally, however there are challenges in early and precise diagnosis due to complexities in characterizing the specific subtype, thus hampering effective prognosis and treatment decisions, thereby increasing demand for novel targets and markers to enhance early breast cancer detection [33]. Proteins from cell lines or tumor cells can be expressed as proteins that fuse with phage coat proteins thus becoming displayed on the surface of phages. Many approaches have been made to design molecular imaging probes including radio-labeled small molecules, monoclonal antibodies and antibody fragments. However, these were deemed clinically ineffective as they had low specificity and insufficient ability to permeate through target tissues [48]. As such, peptides are increasingly used to assist in imaging as they have a high binding affinity, precise cellular uptake, robust stability *in vivo*, swift elimination from non-target areas and enduring presence within the target tissues (Table 2) [48]. Cell penetrating peptides (CPPs) have been able to transport radioisotopes as diagnostic agents such as RGD peptide conjugated with 18F radiolabel agent to target tumors expressing integrin and Cyclic-RGD peptide conjugated with [99mTc(HYNIC-tetramer)(tricine) (TPPTS)] radiolabel agent to target MDA-MB-435 breast cancer cells which express integrin as well [49,50]. Peptides may also have fluorescently quenched fluorophores, peptide linkers that are cleavable and an attached quencher to form optical probes that are activable. Proteolytic cleavage can occur to elevate fluorescence intensity as the quencher ensures little to no signal at the inactivated state, allowing for potential discovery of hidden metastatic lesions [48].

Most peptides obtained from phage display screening used for molecular imaging *in vivo* usually attach to vasculature components as well as proteins with upregulated expression on tumors. Examples include KCCYSL which target HER2 and ANTPCGPYTHDCPVKP which target galectin-3, were used for imaging *in vivo* (Table 2) [51]. The galectin-3 targeting peptide originated from a cysteine-constrained library devised in George Smith's laboratory, effectively hindered interactions between TF and galectin-3 by about 50 %. Its capacity to specifically accumulate in tumor endothelium containing galectin-3 was validated through *in vivo* biodistribution assessments and SPECT imaging studies, utilizing an ^{111}In-DOTA version of the peptide in mice bearing human MDA-MB-435 breast tumors. SPECT/CT examinations utilizing In-DOTA-glysergl

Table 1
Therapeutic peptides developed for screening breast cancer.

Target	Peptide sequence	Library	Mechanism of action	Test method	Peptide affinity (K_d)/LC_{50}/IC_{50}	Reference
HER2	KCCYSL	fUSE5-cpIII phage library	Binds to extracellular domain of HER2	*In vivo* (SCID mice)	K_d = 295 nM	[51]
	PGE/poly(3–3-APPA)/EDC-NHS/Avd/Gly/PEPC3	PD library PhD-12	Bind to luminal breast cancer proteins	*In vitro* (MCF-10 A, MCF-7, MDA-MB-231)	ND	[53]
	PGE/poly(3–3-APPA)/EDC-NHS/Avd/Gly/PEPH2				ND	
C5AR1 (Complement component 5a receptor 1)	CTGNQAAFC	Ph.D.™ – C7C library	Highly stable interaction through a salt bridge with Lys365	*In vitro* (SK-BR-3)	ND	[57]
Insulin receptor			Salt bridge and hydrogen bonds allow for strong interactions			
CX3CR1 (Chemokine (C-X3-C motif) receptor 1)			Hydrogen bonds confer strong interactions			
HER3	CLPTKFRSC	Cysteine-constrained randomized 7-mer library	ND	*In vitro* (MDA-MB-453) *Ex vivo* (Nu/numice)	K_d = 270 ± 151 nM	[58]
Galectin-3	ANTPCGPYTHDCPVKR	f88-Cys6 library	Binds terminal galactopyranose residues on carbohydrates	*In vitro* (MDA-MB-435, PC-3 M)	K_d = 72.2 ± 32.8 nM	[59]
				In vitro (MDA-MB-435) *In vivo* (SCID mice)	IC_{50} = 200.00 ± 6.70 nM	[60]
p53	PGE/poly(3–3-APPA)/EDC-NHS/Avd/Gly/PEPC3	PD library PhD-12	Bind to luminal breast cancer proteins	*In vitro* (MCF-10 A, MCF-7, MDA-MB-231)	ND	[53]
	PGE/poly(3–3-APPA)/EDC-NHS/Avd/Gly/PEPH2		Binds to luminal breast cancer proteins	*In vitro*	ND	
Neuropilin-1	CLKADKAKC	Ph.D.-CX7C™ phage display peptide library	Binds using cryptic C-end rule motif	*In vitro* (MDA-MB-231) *In vivo* (BALB/c athymic nude mice)	ND	[55]
	ATWLPVPVVGYFMASA	fUSE5-cpIII phage library	ND	*In vivo* (Athymic nude mice)	IC_{50} = 16.1 ± 7.7 nM	[61]
"Brain seeking" breast cancer cells	MYPWTEPSYLSN	Ph.D.-12 phage display peptide library	ND	*In vitro* (231-BR) *In vivo* (BALB/c nu/nu mice)	ND	[54]

Table 2
Therapeutic peptides developed for breast cancer treatment.

Target	Peptide sequence	Source	Mechanism of action	Test method	Reference
HER2	KCCYSL	fUSE5-cpIII phage library	Binds to extracellular domain of HER2 (K_d = 295 nM)	*In vivo* (SCID mice)	[51]
	LTVSPWY	Ph.D-7 and Ph.D-12 libraries	Contains common peptide core motif (LTVXPW)	*In vitro* (SKBR3, T47D)	[68]
	WNLPWYYSVSPT		ND		
"Brain seeking" breast cancer cells	MYPWTEPSYLSN-GG-YGRKKRRQRRR-GG-D (KLAKLAK)	Ph.D.-12 phage display peptide library	ND	*In vitro* (MDA-MB-231-BR) *In vivo* (BALB/c nu/nu mice)	[70]
aFGF/FGFRs (acidic fibroblast growth factor/fibroblast growth factor receptors)	AGNWTPI	Ph.D.-7™ Phage Display Peptide Library	Binds to aFGF with electrostatic interactions that may disrupt aFGF-FGFR1 binding	*In vitro* (MDA-MB-231, MCF-7)	[35]
FLAP (5-Lipoxygenase-activating protein)	DPFYSMLQRLAH	12-mer phage-displayed library	ND	*In vitro* (MCF-7)	[71]
Mucin-16	CPTASNTSC EVQSSKFPAHVS	Ph.D.™-C7C and 12-mer phage display peptide library	High homology with the target	*In vitro* (MDA-MB-231, 4 T1)	[66]
PRDM14	DMPGTVLP	Type 8 phage display landscape library f8/8	ND	*In vitro* (MCF-7)	[67]
APaseP (Aminopeptidase P)	CPGPEGAGC	CX7C library	Peptide has 2 X-P-Z sequences, where APaseP cleaves	*In vivo* (ICR CD-1 mice)	[72]

(GSG)-ANTPCG-PYTHDCPVKP reported favorable uptake within the tumor and distinct differentiation in the mice bearing tumors. The binding specificity of the peptide was validated by effectively reducing *in vivo* tumor uptake of ^{111}In-DOTA(GSG)-peptide by 52 % through the introduction of its non-radiolabeled counterpart two hours after injection [51].

Next, KCCYSL is one of the prime instances of a successfully employed tumor imaging peptide derived from phage display which targets HER2 (Table 2). The peptide was isolated from a fUSE5-cpIII phage library of six amino acids generated within the research sphere

of George P. Smith's laboratory. HER2 constitutes a member of the transmembrane receptor tyrosine kinase encompassing the epidermal growth factor receptor family. Although HER2 lacks a recognizable ligand or growth factor, it forms heterodimers with other members of the same family, thus activating diverse signaling pathways leading to escalated adherence, proliferation, angiogenesis and cell viability in tumor cells [51]. HER2 is a significant biomarker for breast and prostate cancer and is a notable target for distinct cancer-specific imaging and therapeutic agents. Antibodies like trastuzumab were designed to target or obstruct HER2 from functioning and have been coupled with various radionuclides for applications in cancer imaging and therapy [51]. KCCYSL, with a binding affinity of 295 nM for the extracellular domain of HER2, was reported to bind to carcinoma cell lines expressing HER2, encompassing ovarian, breast and prostate cancer cell lines. Thus, the peptide potentially emulates a CCY/F motif that exists in EGF-like domains of ligands belonging to the ERBB family. Furthermore, the peptide was linked to DOTA through a GSG spacer and labeled with ^{111}In for Single Photon Emission Computed Tomography (SPECT) imaging of HER2-positive tumors [51]. *In vitro* experiments demonstrated the affinity of ^{111}In-DOTA(GSG)-KCCYSL peptide for MDA-MB-435 human breast cancer cells that express HER2, and competitive studies using non-radioabeled peptide exhibited an IC_{50} value of 42.5 (±2.76) mmol/L. Biodistribution investigations reported swift tumor uptake and complete body clearance of ^{111}In-DOTA(GSG)-KCCYSL in mice bearing human breast carcinoma, and SPECT/CT assessments showed that the distinct visualization of the breast tumor through the radiolabeled peptide conjugate at 2 h after administration. The kidneys were the only non-target organ uptake of the peptide [51]. Overall, this shows that phage display screening of therapeutic peptides is viable especially for breast cancer diagnosis.

Research done by Imai et al. [52] identified that Eph receptor A10, TRAIL-R2 and Cytokeratin 8 expressed in breast cancer tissues are promising candidates for breast cancer biomarkers by incorporating a naive scFv phage library into their antibody proteomics system (Table 2). The study was also able to optimize biopanning protocols using membrane-based panning to isolate antibodies from tiny amounts of antigen extracted from 2D-DIGE spot gel pieces. The system was developed to expedite the screening of potential biomarker proteins through swift generation of cross-reacting antibodies using phage antibody library technology. Through the process established by Imai et al. [52], the isolation of the antibody-expressing phages targeting each of the identified proteins was done within two weeks. Immunostaining of the breast tumor tissue microarrays was subsequently done to obtain the expression patterns. Hence, this demonstrates the effectiveness of phage display libraries in facilitating the discovery of potential biomarker proteins.

Research done by da Fonseca Alves et al. [53] identified recombinant peptides, biotin-C3 and biotin-H2 through phage display library screening against MCF-7 cells and utilized them as biorecognition elements in constructing a biosensor. The peptides effectively differentiated serum samples of breast cancer patients from those with benign breast disease. Additionally, they demonstrated the ability to categorize patients based on the subtypes, distinguishing Luminal A and B tumors through p53 and HER2 expressions respectively. Next, a study by Fu et al. [54] isolated a novel peptide, BRBP1 (MYPWTEPSYLSN) *via in vitro* phage display screening against 231-BR cells, which are human brain-seeking breast carcinoma cells. BRBP1's *in vivo* targeting potential was evaluated through injecting it intravenously into mice with 231-BR tumors (Table 2). Subsequent immunofluorescent staining showed that BRBP1 targeted the tumors as the uptake by normal tissues and organs was limited. It is notable that BRBP1 displayed internalization, thus distributing into the cytoplasm and nucleus after incubating for 2 h. Hence, this peptide not only shows promise for specific brain metastatic breast cancer targeting for early diagnosis, but also shows promise as a potential carrier for anticancer drugs. Another noteworthy breast cancer-targeting peptide known as CK3 (CLKADKAKC) was identified by Feng et al. [55] through phage display. CK3 demonstrated binding specificity to the neuropilin-1 (NRP-1) receptor, a potential breast cancer biomarker correlated with angiogenesis and tumor invasion [56]. This shows that CK3 is a potential diagnostic and therapeutic agent for NRP-1 overexpressed breast cancer. Its biodistribution was analyzed using ^{99m}Tc-labeled CK3 injected into mice with MDA-MB-231 breast tumors. Single-photon emission computed tomography (SPET) and near-infrared fluorescence imaging (NIRF) revealed peptide accumulation in tumors, with moderate presence in kidneys for clearance. Thus, this suggests that SPET and NIRF imaging along with the peptide are potentially clinically applicable for breast cancer, paving the way for new avenues for breast cancer diagnosis and therapeutic response assessment [33].

5. Therapeutic peptides for breast cancer treatment

Peptides have been extensively studied on as an alternative for monoclonal antibodies (mAbs) to circumvent the several disadvantages of mAbs, including dose-dependent toxicity effects upon non-specific uptake by the liver and less ability to be delivered to the tumor cells due to their larger molecular structures thus inhibiting passive diffusion across plasma membranes [48]. Hence, peptides offer reduced non-specific toxicity effects, smaller sizes, more efficient clearance by the kidneys, high specificity as well as better ability to be delivered to tumor cells. The peptides can also be used as radioprobes for tissue-targeted imaging after radiolabeling. Peptides can also accumulate in desired tissues to amplify imaging signals to aid in diagnosis. Anticancer peptides are grouped into 3 main classifications depending on their mechanism of action, which are inhibitory, pro-apoptotic and necrosis-inducing peptides [20,62]. Peptides that are able to bind to integrins expressed on cancer cells will exhibit inhibitory activities, thus limiting the mobility and metastatic potential of the cancer cells. For instance, peptides containing arginylglycylaspartic acid (RGD) exhibit inhibitory effects. These peptides consist of a distinct arginine sequence (Arg-Gly-Asp) and several free radicals, such as benzyl, tert-butyl, diphenylmethyl or 2,2,2-trichloroethyl, integrated into their structure [63]. Hence, these compounds hold considerable potential for the creation of novel RGD-modified conjugates for targeted anti-cancer therapy [20]. Other peptides containing arginine, lysine and histidine residues in their composition possess the ability to permeate tumor cells, thus being known as cell-penetrating peptides. This mode of action offers the opportunity to employ CPPs as delivery systems for therapeutic agents, including peptides that typically have limited permeability. Conjugating therapeutic agents with CPP can induce tumor cell necrosis or apoptosis. The interactions between CPPs and the negatively charged phospholipids and proteoglycans on cell membranes arise due to the presence of positively charged amino acids within CPPs due to electrostatic interactions. Sequences of hydrophobic aliphatic and aromatic amino acid residues further aid in facilitating the penetration of drugs through the lipid bilayer of cell membranes [48].

The peptides that are able to translocate across membranes through direct penetration or endocytosis are known as cell penetrating peptides (CPPs) which ideally are 4 to 30 amino acids long [20]. CPPs have been used as transport systems for the intracellular delivery of antitumor agents, small molecules, imaging molecules, nucleic acids and proteins. It has been reported that CPPs are able to be linked with the desired molecules and translocate across the plasma membrane in a receptor-independent manner, but the mechanisms of uptake of the peptides is dependent on the length, physicochemical properties, concentration and charge of the molecules. In the context of cancer, the overexpression of receptors in tumor cells as compared to healthy cells can facilitate the successful transport of antitumor drugs, liposomes and nanoparticles.

Cell penetrating peptides (CPPs) consist of MPG (N-Methylpurine-DNA Glycosylase) peptides, Pep peptides and TAT peptides [20,64]. Firstly, MPG peptides have three domains with amphiphilic properties, which are hydrophobic, lysine rich and a linker domain. The domains

allow for better targeting, interaction with nuclear substances, uptake, as well as determining the flexibility and integrity of the peptides. Hence, peptides can be used to effectively transport oligonucleotides and plasmid DNA into adherent and non-adherent cells [20,48]. For instance, small interfering RNA (siRNA) are reportedly delivered and rapidly translocate into the nucleus *via* MPG vectors with an efficiency of up to 90 % as the cell entry is facilitated through the interactions of the MPG peptide with the lipids on the cell membrane, thus forming temporary transmembrane alpha helical or beta structures which disrupt membrane organization and subsequently allowing the complex to be inserted into the membrane then translocated from membrane potentials [48].

Next, Pep peptides are non-covalent complexes and amphipathic to circumvent the disadvantage of drug bioavailability as reported in *in-vivo* studies. For instance, Pep-1 peptide contains a hydrophobic domain to facilitate the internalization of small molecules and large proteins while Pep-2 has a modified hydrophobic domain to stabilize the formation of carriers for more rapid uptake [20,48]. Pep-1 also contains aromatic residues to favour disruption of the formation of the cell membrane lipid bilayer. Hence, Pep-3 was designed with the mechanism and allowed for the uptake to be increased up to 92 % [65].

Moving on, TAT (transactivator or transcription) peptides are derived from HIV. It has shown abilities to target cancer such as conjugation with the p53 gene has activated the gene, thus successfully targeting Namalwa lymphoma tumor [48]. Conjugation with Met peptide inhibited hepatocyte growth factor in liver as well. Besides that, colon adenocarcinoma cells in CT26 mice were targeted by chitosan or doxorubicin complexed with TAT to exhibit two-fold higher inhibition when compared to control groups. It was also reported that the ability to target tumors can be increased by having pH sensitive TAT-PEG complexes which are able to release drugs when penetrating cell membranes as tumor cells tend to have acidic pH [48].

A study done by Silva et al. [66] isolated two novel peptides, 4T1pep1 and 4T1pep2 using *in vitro* phage display which demonstrated high binding efficiency and selectivity towards the 4 T1 cell line. The initial biopanning was done using the C7C library, then the sequence of the clones was obtained between rounds to allow for analysis of obtained peptide hits. The study identified 5 different peptides, but 4T1pep1 (CPTASNTSC) showed a 100 % enrichment, prompting its selection for further analysis. Subsequent biopanning using the 12-mer library necessitated phage amplification due to titer loss in the previous rounds. DNA sequencing of the individual clones from the final round revealed identical sequences with 100 % similarity. Despite minimizing biased sequences using a specific *E. coli*$^{+}$ strain (JM109), potential non-specific targets from materials in the biopanning process need to be discarded as well as identifying false positives. Hence, bioinformatics was used where web-based tools like PepBank and SAROTUP can aid in searching for reported peptides or unintended material-binding peptides. PepBank analysis reported no significant similarity with reported peptides thus reinforcing the novelty of the peptides. Immunofluorescence assay was subsequently conducted, where the peptides had positive binding to the 4 T1 cell line and to human MDA-MB-231 cells. The study also found that mucin type isoform (Mucin-16) hit protein is potentially involved in the underlying mechanisms behind breast cancer carcinogenesis. The study established the groundwork for the translational potential of the peptides in cancer therapy, particularly nano-based targeted delivery for TNBC therapy [66]. This further shows the promising ability of phage display screening for novel peptides.

Furthermore, phage-derived peptides were used in a study by Bedi et al. [67] to enhance the potential effectiveness of liposome-encapsulated siRNAs for anticancer purposes. The phage, named DMPGTVLP after the structure of the peptide which exhibits affinity towards breast cancer cells, was selected from a vast multibillion-clone landscape phage library called f8/8. The primary coat protein of the selected phage was transformed into liposomal vesicles loaded with the drug; thus the phages span the lipid bilayer to display tumor-targeting peptides on the vesicle surface. The siRNA-phage fusion protein liposomes were able to effectively shield PRDM14 gene-targeted siRNA indicated by PicoGreen fluorescent assay. Besides, the liposomes maintained their size and size distribution in 10 % serum throughout a week which indicates their stability to be potentially used as treatments. PRDM14 gene expression was successfully downregulated and PRDM14 protein synthesis was hindered in MCF-7 cells through this approach, with the efficacy being suggested to be comparable to the gold-standard lipofectamine-siRNA complex for *in vitro* delivery into cells. The authors also reported that the siRNA-liposomes alone did not influence PRDM14 gene expression, thus implicating that fusion proteins play roles in cell targeting, anchoring and mediating siRNA-liposome delivery into cells [67]. Thus, this demonstrates the capability of phage-derived peptides in future clinical applications for breast carcinoma.

Shadidi and Sioud [68] utilized commercial M13-based 7- and 12-mer phage libraries to identify internalizing peptides specific to breast cancer cells. These peptides are characterized by their ability to penetrate cells upon receptor recognition, thus allowing the delivery of conjugated active agents into the target cells' cytoplasm. Two peptide motifs were identified after multiple rounds of panning on the HER2-positive SKBR3 breast cancer cell line, the motifs being LTVSPWY and WNLPWYYSVSPT. The second peptide was also found to be able to bind to other cancer types which are glioma, colon, lung and prostate cancer cells. The peptide was reported to internalize thus delivering fluorescein-conjugated anti-HER2 antisense oligonucleotides into SKBR3 cells [68]. Hence, specific delivery of therapeutic agents can be achieved as indicated by the inhibition of HER2 gene expression.

Research by Wang et al. [69] used an 8-mer landscape phage library to obtain a phage fused with a peptide specific for MCF-7 cells. The phage fusion proteins self-assembled with liposomes loaded with doxorubicin which reported an increase in cancer cell binding and induced tumor cell destruction. They also assessed the efficacy and potential toxicity of the phage *in vivo* where subcutaneous, and orthotopic xenografts of human breast cancer tissues were utilized. There was favorable response as the tumor displayed significant remission in comparison to control non-targeted formulations in the xenografts with a notably shorter time of onset of tumor reduction as well. The antitumor activity was shown to be increased by the phage as evidenced by the observation of necrotic areas containing the least amount of viable tumor cells within the tumors targeted by the phage-Doxil conjugate. This was caused by the phage-Doxil conjugate facilitating nuclear delivery and accumulation of the drugs within the tumor cells. Fortunately, there were no detectable toxicity effects on the liver indicated by the measurement of plasma ALT and AST levels remaining within normal levels when compared to the untreated control groups [69]. Hence, combining the passive targeting of liposomal nanocarriers along with tumor cell recognition and internalization systems show potential for targeted drug delivery of antitumor drugs *in vivo*.

6. Challenges and future perspectives

It can be observed that phage display screening for therapeutic peptides has brought many advantages especially in the context of breast cancer diagnosis and therapeutics. The peptides derived from phage display are particularly specific for desired targets, vast application approaches, easily produced and modified, as well as implicated to have less toxic side effects by being specific to target tissues. Peptides derived from phage display screening have reported promising results for *in vitro* and *in vivo* studies. Nevertheless, this technology has challenges to overcome for the wide clinical application of peptides.

A challenge faced by phage display libraries is that the quality and variety of peptides depend on the source of the library, the diversity of the library used, and the screening procedures utilized [73]. Another challenge is to increase the avidity of peptides, which refers to the ability to bind to the target through multiple interactions, thus amplifying

binding strength and increasing the residence time of the target molecules at the binding site to allow for a high local concentration [74]. This is because peptides tend to have high affinities towards their target which is the strength of the interaction between a peptide and a ligand but exhibit lack of avidity. Hence, researchers often decorate the surface of nanocarriers with short linear peptides with the aim to increase the chances of interaction with targeted ligands. For example, the NGR peptide was fused with TNFα, a cytokine with high systemic toxicity which caused it to have limited clinical use. This effectively reduced the cytokine's severe side effects as it exhibited effectiveness at doses 1000 times lower than the usual dose. This can be attributed to the fact that the TNFα has a quaternary structure which forms a trimer, thus the NGR peptide will attach to each subunit to form a three-to-one ratio of NGR peptide to TNFα. Hence, this increased receptor binding due to increased avidity [74].

Another challenge of using peptides is that peptides containing 5 amino acids and less would be water soluble with the solubility reducing as the length of the peptide increases. Phage display derived peptides would produce peptides containing between 7 and 30 amino acids. Hence, the peptides may form specific 3-dimensional structures which induce precise binding to the receptors. However, it is a challenge to properly solubilize the peptides as it is important to avoid improper solubilization to avoid loss of peptide activity [74]. Research conducted by Xiao et al. [75] utilized a method where they attached betaine molecules onto bacterial xanthine guanine phosphoribosyltransferase protein and the HIV inhibitory peptide, producing promising results that conjugating betaine would be able to mitigate peptide aggregation as well as enhancing the solubility of phage-derived peptides.

Furthermore, some phage display libraries constructed contain peptides exhibited on the surfaces of phages which have not been modified and the original conformation of these peptides may differ to an extent. Hence, these constructed libraries may not be able to accurately reflect the original conformations of peptides *in vivo*. This means that even if a screen peptide may exhibit binding affinity, it may not necessarily act as an antagonist nor have therapeutic function. For instance, four peptides (AV1, AV2, AcBl3 and AcB14) were found to exhibit affinity towards 3F (SEMA3F/plexin-A2) but were unable to be used in animal models. Hence, this indicates that it is important to enhance screening techniques from knowledge gained from experiences. Researchers have also combined phage display with other methods including high-throughput sequencing to provide more comprehensive understanding and categorization of phages after screening [76]. For example, research by Nisticò et al. [77] reported that whole cell-based screening on a TNBC cell line that overexpresses epidermal growth factor receptors (EGFR), may possibly preserve the native folding and the post translational modifications, as compared to screening using purified EGFR protein or extracellular domains, providing precious insight into methods to mitigate the limitations of phage display.

Due to the limitations raised throughout the years of research utilizing phage display screening technology, alternative display techniques have emerged. For instance, yeast display was possible by incorporating the protein of interest into the surface glycoproteins on the yeast cell wall. Yeast display was mainly used due to it being a eukaryotic display system, thus maintaining eukaryotic post-translational modifications, which are absent in phage display. Thus, yeast display is particularly advantageous for applications requiring protein folding and modification, such as the engineering of antibodies, including antibodies against antihuman immunodeficiency virus (HIV)-1 and glioblastoma stem-like cells [78,79]. However, yeast display has smaller library sizes containing up to 107 individual clones as compared to phage display techniques having a library size of 109 individual clones, hence limiting the application of yeast display for high-throughput screening [79].

Ribosome display was investigated to address the limitation of phages by allowing the screening of larger libraries consisting of up to 1012–1014 members [78]. This display method requires a protein library encoded within a DNA to be transcribed into mRNA then purified and undergo cell-free translation *in vitro*. Next, mRNA-ribosome-peptide complexes underwent affinity selection, from there, the mRNA dissociates, and reverse transcribed into cDNA. This cDNA will be amplified, enriched, sequenced and analyzed using PCR. As such, Bacterial transformation was not required for ribosome display, allowing for the production of proteins that were sensitive to proteases as well as unstable proteins. This is however limited by risk of RNase contamination and the inherent affinity of mRNA or ribosomes for target molecules, thus competing with the binding of displayed peptides or proteins [78].

Another alternative is mRNA display, where a DNA library is constructed, followed by *in vitro* transcription into mRNA in a cell-free environment. Protein-mRNA complexes are formed through the *in vitro* cell-free translation of mRNA. Then, a short DNA-puromycin linker on the 3′ end of the transcribed mRNA, allows for a covalent connection to the displayed protein, producing a complex that is more stable than ribosome display [78]. This technology also reduces the likelihood of interacting with an immobilized selection target due to the smaller size of the puromycin DNA linker as compared to a ribosome, thus producing less biased results than ribosome display. However, mRNA display technology is more complex and requires additional steps for the stabilization of mRNA-protein fusions. Research by Hurd et al. [80] utilized a random non-standard peptide integrated discovery system to increase the scope of screenable targets in mRNA display without requiring prior purification, but may not be applicable to all protein targets. This technology is still advantageous over phage display with regards to highly post-transcriptionally modified proteins, which is not available in phage display screening [80]. Applications of mRNA display include the identification of macrocyclic peptides and protein-protein or DNA-protein interaction mapping [78,81,82].

Overall, there have been many advancements made in the field of peptide screening technologies, and each technology brings about their own unique advantages as well as their own limitations that can potentially complement the disadvantages of phage display. As such, this could possibly lead to novel hybrid approaches in peptide screening. For instance, the combination of yeast surface display and phage display to select peptides that inhibit amyloid fibril formation, as unstable proteins can be stabilized and expressed on the yeast surface, while phage display allowed for a wide selection of peptides [83]. Other than that, emerging computing technologies allow for more robust computer-aided phage display methodologies by offering secondary optimization strategies. This involves bioinformatics analysis, molecular docking, machine learning (ML) and artificial intelligence (AI) to further facilitate the identification and evaluation of potentially novel anticancer peptides targeting receptors of interest in breast cancer therapeutics [84,85].

Recent breakthroughs in the integration of phage display with AI have remarkably developed peptide selection for diagnosis and treatment against breast cancer [86]. Most importantly, AI-driven algorithms have been employed, particularly machine learning models, to analyze large datasets generated from phage display libraries for identifying high-affinity peptides with enhanced specificity for tumor biomarkers such as HER2 and estrogen receptors [44]. This approach not only accelerates the identification of novel peptides but also refines their specificity by predicting peptide-protein interactions, thereby optimizing peptide sequences with minimal off-target effects. For instance, AI can classify and prioritize peptides based on their binding affinities in a high-throughput manner to allow researchers to focus their resources on the most promising candidates for further experimental validation [87]. This combination of phage display and AI provides a powerful tool for the development of personalized cancer therapies, with possible peptide tailoring even to an individual molecular profile of the disease. Notably, the improvement of computational tools enabled the incorporation of AI into the process of peptide screening, which enhanced the efficacy of searching for potential therapeutic agents [88,89].

Computational methodologies such as AI and machine learning will

significantly enhance the screening of phage libraries in a high-throughput manner by analyzing large volumes of data for the identification of peptides with the highest binding affinity and specificity. Machine learning models predict peptide-protein interactions and optimize peptide sequences, thus helping in the identification of more promising candidates for further testing [87]. In addition, such tools will save time-consuming and costly manual screening by underlining peptides that most presumably would be effective for a certain personalized treatment. That's where the integration of AI expedites the development of novel therapeutic peptides for individual patient profiles and increases precision as well as efficiency in personalized treatments for cancer [89].

Future research should focus on strategies to utilize it clinically as many peptide-based targeting ligands have exhibited promising results to aid cancer therapeutics through mechanisms such as increased drug accumulation, targeting tumors with high specificity and induced inhibition effect on tumors when conjugated or used together with antitumor drugs or biologics. To facilitate successful translation in clinical settings, the peptide-targeting ligand requires optimization for affinity, avidity, target specificity and solubility in water. This is because although biopanning is effective, it requires careful optimization of a variety of parameters to yield specific peptides that have high affinity as well. Factors such as the type of phage display library, concentrations of phages and antigens required, and incubation and elution conditions will require careful evaluation. Selecting between *in vitro*, *in situ*, or *in vivo* affinity selection will also significantly impact the stability, selectivity, biodistribution and clearance of both peptides and peptide-conjugated nanoparticles [90].

The peptides will require modifications as well to enhance membrane permeability while avoiding disrupting biologically active conformations of the peptides, maintaining stability against metabolic processes including intestinal, plasma and cellular proteases, activities of intestinal and hepatic cytochrome P450 enzymes and P-glycoproteins [91]. It is noted that the rapid clearance rates of peptides should be reduced as well. As such, the route of administration is crucial. For instance, peptides delivered through parenteral administration would often require frequent dosing to maintain therapeutic levels and are at risk of degradation by proteases and peptidases. Oral administration of peptides would also need to overcome the challenges of the harsh conditions of the gastrointestinal tract and absorption into the bloodstream. Buccal delivery is a promising alternative as the peptide can directly be absorbed into the bloodstream but may be unpleasant or inconvenient for patients [90].

Future research should focus on developing methods to optimize the delivery and transport of peptides which have shown promising results in prior research, such as conjugating with PEGs or lipids to avoid degradation and change in conformation of the peptides [91]. Nanotechnology has shown promising results due to their scale ranging from 1 to 100 nm, enhancing permeability and retention effects which is particularly desirable for the delivery of high concentrations of contrast agent to targeted tumor sites. There is also a high surface area to volume ratio for increased surface functionality and degree of modification to improve the binding affinity of individual probes [33,92]. As such, research utilizing nanoparticles modified with peptides derived from phage display may improve drug delivery due to the high affinities of peptides towards receptors of interest, increasing cytotoxicity of existing drugs towards breast cancer [86].

Several challenges exist in the clinical translation of phage display-derived peptides. One of the main challenges involves regulatory hurdles and the complexity of obtaining FDA (U.S. Food and Drug Administration) approvals, as therapeutic peptides must undergo rigorous testing to ensure their safety and efficacy in human use. Scaling up peptide production from laboratory to commercial levels presents another challenge, as the manufacturing process must maintain consistency and quality while being cost-effective. Additionally, optimizing peptides for human use requires overcoming issues such as their rapid degradation in the bloodstream, short half-lives, and potential off-target effects [63]. A clear example of the efforts towards clinical translation is the ongoing development of phage display peptides in preclinical trials, such as HER2-targeting peptides that are currently being evaluated for their ability to treat HER2-positive breast cancer patients. These peptides have shown promising results in animal models, but further optimization and larger clinical studies are necessary before they can be widely used in clinical settings [74].

A detailed pathway from preclinical studies to clinical application begins with extensive biomarker validation, ensuring that the peptides are targeting specific cancer effectively. After preclinical testing, potential peptides enter phase I clinical trials to evaluate their safety, dosage, and potential side effects in small groups of patients. After successful completion of phase I, phase II trials focus on peptide efficacy by testing the peptides in larger groups of patients with the targeted condition. Phase III trials further assess the peptides' effectiveness compared to standard treatments. Throughout this process, peptide optimization is crucial, involving modifications such as PEGylation to increase their stability, reduce toxicity, and enhance bioavailability [90]. Fusion proteins can also be explored to enhance peptide delivery and activity in the human body, offering a pathway to create more effective therapeutic agents. By addressing these barriers and optimizing the peptide properties, phage display-derived peptides can move closer to becoming reliable treatment options for cancer patients [32].

7. Conclusion

Phage display has been gaining attention due to its effectiveness in various biological science fields, especially within cancer therapeutics and diagnosis. Peptides derived from phage display have been shown to exhibit high affinities and specificities towards proteins highly associated with breast cancer such as HER2 receptors. Peptides are able to circumvent challenges of using antibodies which consist of larger sizes, a slower clearance rate, a higher risk of immunogenicity, limited ability to penetrate tumors and higher uptake by the liver. Peptides, when compared to antibodies, also cost less to manufacture, offer greater stability, minimal immunogenicity thus having negligible toxicity, as well as being easy to be modified such as through conjugation with liposomes or antitumor drugs. Phage display technology is usually utilized to isolate peptides of interest by screening a large amount of targeted samples at the same time. Many peptides derived from phage display are tumor targeting peptides and cell-penetrating peptides, which are specific for tumor cells and have been researched extensively on to improve their potential in clinical applications, such as conjugating with fluorescence probes to aid in molecular imaging and conjugating with antitumor drugs to inhibit tumor cells through mechanisms such as drug accumulation. There is much research implicating the potential of phage-derived peptides to aid in diagnosis and treatment of breast cancer *in vitro*, hence research should focus on applying this knowledge *in vivo* as well as humans in clinical settings. Approaches such as radiolabeled peptides and conjugation with liposomes or drugs have shown immense potential. The challenges of using phage-derived peptides will require modifications or isolation of peptides with higher water solubility, higher avidity, optimized membrane penetration ability without compromising the biologically active conformations of the peptide and have the ability to maintain stability in the presence of proteases in the body. It is also highly recommended that phage display technology be combined with computational methods such as artificial intelligence (AI) to potentially produce more optimized peptides efficiently. Nonetheless, phage display technology has shown huge potential in isolating promising peptides to target breast tumors for diagnostics and therapeutics, paving the way for accelerated development within personalized medicine for breast cancer.

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CRediT authorship contribution statement

Ashlyn Wen Ning Yau: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Sylvester Yee Chun Chu:** Writing – original draft, Investigation, Formal analysis, Data curation. **Wei Hsum Yap:** Writing – review & editing, Validation, Supervision. **Chuan Loo Wong:** Writing – review & editing, Validation, Supervision, Formal analysis. **Adeline Yoke Yin Chia:** Writing – review & editing, Validation, Supervision. **Yin-Quan Tang:** Writing – review & editing, Validation, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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