DEVELOPMENT OF A MESENCHYMAL STEM CELL-BASED SIGNAL PATHWAY DELIVERY PLATFORM FOR TARGETED THERAPY OF HER2-POSITIVE TUMOUR

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PEMBANGUNAN PELANTAR PENGHANTARAN LALUAN ISYARAT BERASASKAN SEL STEM MESENKIMA UNTUK TERAPI BERSASAR TUMOR HER2-POSITIF

by

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LIST OF SYMBOLS

α	Alpha
γ	Gamma
nm	Nanomolar
℃	Degree Celsius
hr	Hour
µg	Microgram
(+)	Positive
mg	Milligram
kg	Kilogram
{}	Miller Index
µl	Microliter
ml	Milliliter
µm	Micrometer
min	Minutes
%	Percentage
~	Approximately
(+)	Positive
pH	Potential of Hydrogen
-	Negative

LIST OF ABBREVIATIONS

BAX	Bcl-2-associated X protein
BCL-2	B-cell lymphoma 2
CapeOx	Capecitabine + Oxaliplatin
CAR-T	Chimeric Antigen Receptor T-cell
CCFC	Cu+-contained Cu/Fe Nanocrystal Clusters
CCFCB	BSA-modified CCFC
CCL4	Chemokine (C-C motif) ligand 4
CD105	Cluster of Differentiation 105
CD11b	Cluster of Differentiation 11b
CD19	Cluster of Differentiation 19
CD3	Cluster of Differentiation 3
CD34	Cluster of Differentiation 34
CD4	Cluster of Differentiation 4
CD45	Cluster of Differentiation 45
CD8	Cluster of Differentiation 8
CDK4	Cyclin-Dependent Kinase 4
CDK6	Cyclin-Dependent Kinase 6
C-fos	Cellular FBJ Osteosarcoma Oncogene
C-jun	Cellular Jun Proto-oncogene
C-myc	Cellular Myelocytomatosis Oncogene
COX-2	Cyclooxygenase-2
CTLs	Cytotoxic T Lymphocytes
CVD	Cardiovascular Disease
EDS	Energy Dispersive X-ray Spectroscopy
EPR	Electron Paramagnetic Resonance
ER81	ETS Related Protein 81
ERK	Extracellular Signal-Regulated Kinase
FAS	Fatty Acid Synthase
FDA	Food and Drug Administration
FFT	Fast Fourier Transform
GAL4	Galactose 4

GAL4	Galactose 4
GCV	Ganciclovir
GJC	Joint and Muscle Pain
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GSH	Glutathione
HE	hematoxylin-eosin staining
Her1	Human Epidermal Growth Factor Receptor 1
Her2	Human Epidermal Growth Factor Receptor 2
Her3	Human Epidermal Growth Factor Receptor 3
Her4	Human Epidermal Growth Factor Receptor 4
HLA-DR	Human Leukocyte Antigen - DR isotype
hPBMCs	Human Peripheral Blood Mononuclear Cells
HRPC	High-risk Prostate Cancer
HRTEM	High-Resolution Transmission Electron Microscopy
HSV-TK	Herpes Simplex Virus Thymidine Kinase
HSV-TK	Herpes Simplex Virus Thymidine Kinase
hTERT	Human Telomerase Reverse Transcriptase
iDFS	Invasive Disease-free Survival
$iMSC^{Endostatin}$	Intelligent Drug Delivery Platform (Secretory drug release)
Ki-67	Marker of Proliferation Ki-67
MAPK	Mitogen-Activated Protein Kinase
MBC	Metastatic Breast Cancer
MEK	Mitogen-Activated Protein Kinase Kinase
Mmp-2	Matrix Metalloproteinase-2
Mmp-9	Matrix Metalloproteinase-9
MMPs	Matrix Metalloproteinases
MSC	Mesenchymal Stem Cells
MSC	Mesenchymal Stem Cells
$MSC^{intelligent}$	Intelligent Drug Delivery Platform (Ruptured Drug Release)
NEC	Notch Extracellular Region
NICD	Notch Intracellular Domain
NMPA	National Medical Products Administration
Notch (core)	Notch core area
p21	Cyclin-Dependent Kinase Inhibitor 1

p53	Tumour Protein 53
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-Kinase
RAF	Rapidly Accelerated Fibrosarcoma
Ras	Rat Sarcoma
ROS	Reactive Oxygen Species
Sp1	Specificity Protein 1
TNM	Tumour-Node-Metastasis
ToGA	Trastuzumab for Gastric Cancer
TyTAN	Tianjin Youth Talent Activity Network
UAS	Upstream Activating Sequence
UAS	Upstream Activating Sequence
VEGF	Vascular Endothelial Growth Factor
VP64	Viral Protein 64
WHO	World Health Organization
XRD	X-ray Diffraction

LIST OF APPENDICES

PEMBANGUNAN PELANTAR PENGHANTARAN LALUAN ISYARAT BERASASKAN SEL STEM MESENKIMA UNTUK TERAPI BERSASAR TUMOR HER2-POSITIF

ABSTRAK

Tumor Her2 (+) mempunyai ciri-ciri seperti migrasi yang kuat, kepekaan rendah terhadap ubat kemoterapi, pemulihan yang teruk, mudah berulang, dan tahap keganasan yang tinggi. Walau bagaimanapun, kaedah tradisional rawatan berfokus sering terganggu oleh halangan biologi yang mengasingkan molekul ubat dalam tisu sihat dan menyebabkan kerosakan pada sel normal. Kajian ini telah mereka bentuk platform penghantaran pintar dengan memperkenalkan laluan isyarat buatan ke dalam sel stem mesenkima (MSC). Platform penghantaran pintar ini berbeza daripada tumpuan tradisional pada mikropersekitaran tumor atau meningkatkan afiniti dengan tumor, dengan membekalkan platform penghantaran dengan keupayaan pengenalan isyarat tumor, membolehkannya membezakan antara tumor dan sel tisu normal serta memberikan arahan penyuntingan maklum balas. Secara khususnya, laluan isyarat buatan MSC dibahagikan kepada dua bahagian: struktur pengenalan dan struktur tindak balas, yang masing-masing memainkan peranan dalam mengenal pasti tumor dan bertindak balas terhadap isyarat tumor. Untuk membolehkan MSC melepaskan ubat dengan cekap, kajian ini melengkapkan MSC dengan laluan isyarat buatan Affibody-Notch (teras)-VP64-GAL4/UAS-HSV-TK dan membina platform penghantaran pintar untuk pelepasan ubat terkawal (MSCintelligent). Gen bunuh diri HSV-TK dalam laluan isyarat ini boleh pecah dan mati di bawah tindakan gansiklovir (ubat bertoksisiti rendah untuk rawatan klinikal herpes), dengan itu melepaskan ubat yang dibawa. Dalam kajian ini, dengan

mengambil MSCintelligent yang dimuatkan dengan gugusan nanohablur Cu/Fe yang diubah suai dengan BSA (CCFCB) sebagai contoh, sistem ko-kultur telah diwujudkan antara MSCintelligent dan sel tumor atau sel tisu sihat. Hasil pengimejan pendarfluor, sitometri aliran, dan pengimejan stesen kerja sel hidup semuanya menunjukkan bahawa MSCintelligent dapat mengenal pasti sel tumor dengan tepat daripada sel tisu sihat dan melengkapkan kesan anti-tumor. Walau bagaimanapun, kaedah pelepasan ubat terkawal ini bergantung pada kematian platform penghantaran itu sendiri, yang akan membawa kepada kekurangan ketekalan dan kesinambungan dalam proses rawatan ubat. Di samping itu, MSCintelligent mungkin lebih sesuai untuk membawa ubat tersedia. Seterusnya, dengan menambah baik laluan isyarat buatan dalam MSCintelligent dan menggunakan endostatin sebagai contoh, platform penghantaran pintar untuk pelepasan ubat yang berterusan telah dibina (iMSCEndostatin) dengan melengkapkan MSC dengan laluan isyarat buatan Affibody-Notch (teras)-VP64-GAL4/UAS-Endostatin. Hasil kajian in vivo dan in vitro menunjukkan bahawa iMSCEndostatin dapat mengenal pasti sel tumor Her2 (+) dengan tepat dan secara berterusan serta efisien mengeluarkan endostatin selepas pengenalan, lalu memberikan kesan anti-tumor. Secara kesimpulannya, kaedah pengagihan ubat eksklusif platform penghantaran pintar ini dapat menyasarkan rawatan di kawasan tumor dan mengurangkan kesan sampingan ubat.

DEVELOPMENT OF A MESENCHYMAL STEM CELL-BASED SIGNAL PATHWAY DELIVERY PLATFORM FOR TARGETED THERAPY OF HER2-POSITIVE TUMOUR

ABSTRACT

Her2 (+) tumours have the characteristics of strong migration, poor sensitivity to chemotherapy drugs, poor recovery, easy recurrence, and high malignancy. However, traditional methods of targeted treatment are often hindered by biological barriers, which isolate drug molecules in healthy tissues and cause damage to normal cells. Here, the study designed an intelligent delivery platform by introducing artificial signal pathways to mesenchymal stem cells (MSC). This intelligent delivery platform is different from traditional targeting of tumour microenvironment or enhancing affinity with tumours, endowing the delivery platform with tumour signal recognition ability, enabling it to autonomously distinguish between tumours and normal tissue cells, and feedback editing instructions. Specifically, the artificial signalling pathway of MSC is divided into two parts: recognition structure and response structure, which respectively play a role in identifying tumours and responding to tumour signals. To enable MSC to efficiently release drugs, this study endowed MSC with Affibody-Notch (core)-VP64-GAL4/UAS-HSV-TK artificial signalling pathway and constructed an intelligent delivery platform for controllable drug release ($MSC^{intelligent}$). The HSV-TK suicide gene in this person's signalling pathway can rupture and die under the action of ganciclovir (a low-toxicity drug for the clinical treatment of herpes), thereby releasing the loaded drug. In this study, taking $MSC^{intelligent}$ loaded with BSA-modified Cu/Fe nanocrystal clusters (CCFCB) as an example, a co-culture

system was established between MSC intelligence and tumour or healthy tissue cells. Fluorescence imaging, flow cytometry, and live cell workstation imaging results all showed that $MSC^{intelligent}$ can accurately distinguish tumour cells from healthy tissue cells and complete anti-tumour effects. However, this controllable drug release method relies on the death of the delivery platform itself, which will lead to a lack of persistence and continuity in the drug treatment process. Moreover, $MSC^{intelligent}$ may be more conducive to carrying finished drugs. Subsequently, by improving the artificial signaling pathway in $MSC^{intelligent}$ and used endostatin as an example to construct an intelligent delivery platform for sustainable drug release ($iMSC^{Endostatin}$) by endowing MSC with Affibody-Notch (core)-VP64-GAL4/UAS-Endostatin artificial signaling pathway. Both *in vivo* and *in vitro* results indicate that $iMSC^{Endostatin}$ can accurately recognize Her2 (+) tumour cells and continuously and efficiently secrete endostatin after recognition, thereby exercising its anti-tumour effect. In summary, the exclusive drug deployment method of the intelligent delivery platform can lock treatment at the tumour site and reduce drug side effects.

CHAPTER 1

INTRODUCTION

1.1 Introduction

In the process of tumour treatment, therapeutic drugs must overcome a variety of biological barriers before entering the target tumour tissue, including intravascular barrier, endothelial barrier, extracellular barrier and cellular barrier (Kim et al., 2020). These barriers will prevent drug molecules from reaching the tumour site, expose them to healthy tissues, and lead to inevitable side effects (Figure 1.1) (Zhao et al., 2020).

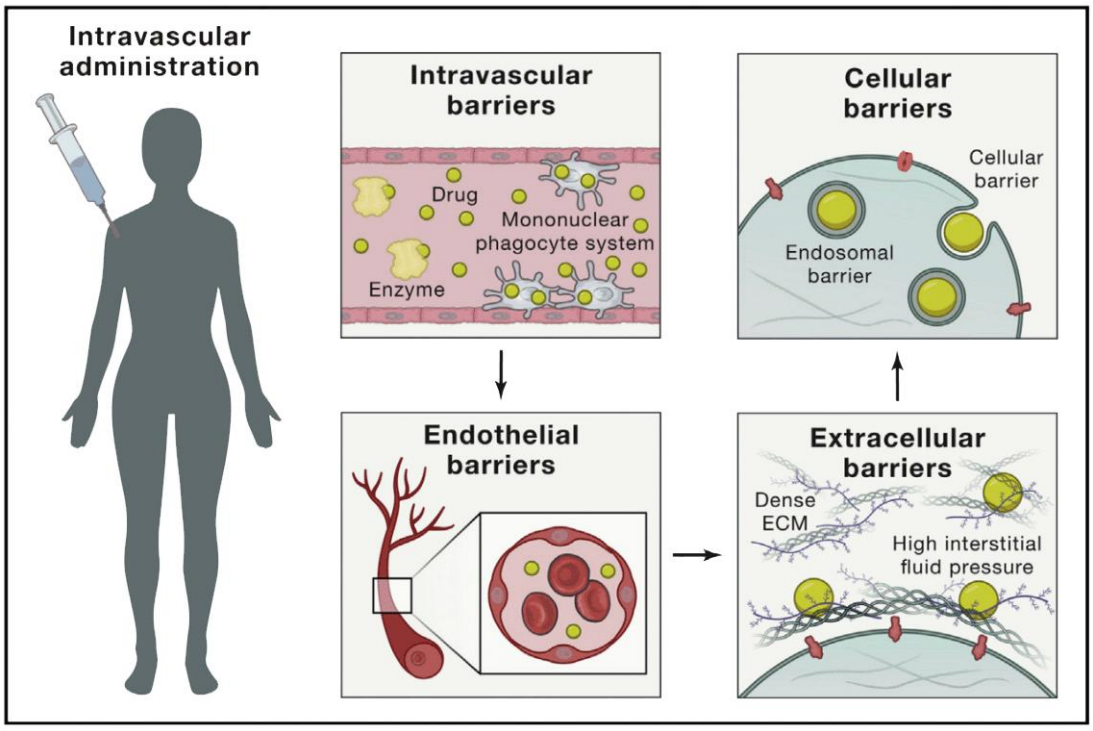

Figure 1.1 Biological barriers for tissue-specific drug delivery intravascularly administered therapeutics encounter serial biological barriers including intravascular barriers, endothelial barriers, extracellular barriers, and cellular barriers (Zhao et al., 2020).

The intravascular barrier is one of the primary obstacles in the delivery process of therapeutic drugs, and its complexity and diversity directly affect the

effectiveness and bioavailability of drugs. In blood vessels, therapeutic drugs may be influenced by various biological and physiological factors, including endovascular enzymes, renal filtration, etc., which limit the stability and activity of drugs in the body (Rossin et al., 2018). Firstly, endovascular enzymes, including proteases and nucleases, have the ability to degrade active ingredients, thereby reducing the activity and efficacy of drugs (Blanco et al., 2015). This degradation process may occur shortly after the drug enters the circulatory system, making it difficult to reach the target tissue or cells effectively. In addition, renal filtration is also an important biological barrier that therapeutic drugs encounter in the blood vessels (Gustafsonet al., 2015). Small-molecule (<6 nm) drugs and large-molecule drugs of a certain size may be filtered and cleared by the kidneys, thereby limiting the residence time and bioavailability of drugs in the body (Gustafson et al., 2015). This clearance process mainly depends on the molecular size and polarity of the drug, while macromolecular drugs and nanoparticles are more easily captured and cleared. In addition to these biological factors, some physical factors can affect the permeability of the intravascular barrier (Blanco et al., 2015; Gustafson et al., 2015). For example, the speed of blood flow and fluid dynamics have a significant impact on the distribution and residence time of drugs (Gustafson et al., 2015; Zhao et al., 2020). Rapid blood flow can quickly bring drugs into the systemic circulation, but it can also limit the residence time of drugs in target tissues or cells. On the contrary, slow blood flow can increase the residence time of drugs in the target tissue, but it also increases the risk of drug degradation or clearance in the blood , thereby reducing the efficacy of drugs (Blanco et al., 2015; Gustafson et al., 2015).

When drugs cross the intravascular barrier, they encounter the endothelial barrier, which significantly influences their permeability and distribution (Xu et al.,

2021). The endothelial barrier is composed of closely packed endothelial cells, forming a critical part of the vascular wall, primarily found in blood vessels and capillaries. This barrier's primary function is to restrict the entry of external substances and cells, thereby protecting the stability of the intravascular environment and maintaining endothelial cell function. (Banks, 2016). The connections between endothelial cells create a structure known as tight junctions, which confer a high degree of continuity and integrity to the endothelial layer. These tight junctions restrict the free diffusion of solutes and cells across the vascular wall, thereby limiting the penetration of therapeutic drugs into tissues or organs. Additionally, specific proteins on endothelial cells, such as integrins and selectins, play crucial roles in cell-cell and cell-matrix interactions, regulating endothelial permeability barrier (Blanco et al., 2015). Moreover, endothelial cells secrete various cytokines and signalling molecules to regulate the stability of the vascular environment and maintain the function of the vascular wall (Xu et al., 2021). These cytokines include some pro-angiogenic factors and anti-angiogenic factors, which have important effects on the proliferation, migration and differentiation of vascular endothelial cells, and then affect the permeability and integrity of the endothelial barrier (Blanco et al., 2015; Banks, 2016). In addition, the permeability of the endothelial barrier may be different in different tissues and organs. For example, a blood-brain barrier is a special form of endothelial barrier, which has very high permeability and selectivity, making most drugs unable to pass through (Rosenblum et al., 2018). In contrast, the endothelial barrier of other tissues and organs may be relatively low, making it easier for drugs to penetrate (Yuan et al., 2012). Finally, some disease states may affect the function and integrity of the endothelial barrier. For example, inflammation and infection may lead to the damage and destruction of endothelial cells, which

increases the permeability of the endothelial barrier and makes drugs easier to penetrate (Xu et al., 2021). On the contrary, some diseases such as tumours may lead to the proliferation and enhancement of endothelial cells, which aggravates the limitation of the endothelial barrier and makes it difficult for drugs to penetrate (Zhao et al., 2020).

The extracellular barrier is the obstacle that drugs encounter in the extracellular matrix before they can reach the lesion (Subrahmanyam & Ghandehari, 2021). The extracellular matrix barrier not only provides structural support for cells but also plays a crucial role in regulating cell behavior, signal transduction, and cell-cell interactions (Blanco et al., 2015). During the delivery of therapeutic drugs, the extracellular matrix serves as a biological barrier that directly affects the diffusion, distribution, and biological activity of drugs (Ilahibaks et al., 2024). The structure of the extracellular matrix is complex and diverse, including components such as collagen, fibronectin, proteoglycans, etc (Zhao et al., 2024). These components not only form scaffold structures but also participate in signal transduction and cell-cell interactions. This complex structural characteristic leads to different permeability and barrier characteristics of the extracellular matrix in different tissues and organs, which has a significant impact on the permeability of therapeutic drugs. The composition and structure of the extracellular matrix can affect the distribution and residence time of therapeutic drugs in tissues (Maisel, 2021). Compared to small molecule drugs, the diffusion of large molecule drugs and nanoparticles in the extracellular matrix is more restricted, which may lead to uneven distribution of drugs in tissues, thereby affecting their therapeutic effects. In addition, some disease states such as tumours and inflammation may cause changes in the structure and composition of the extracellular matrix, thereby affecting the diffusion

and distribution of drugs (Xu et al., 2022). Subsequently, the cellular barrier generated by intercellular interactions and endocytosis can also affect the activity of drugs (Zhao et al., 2019).

These biological barriers can restrict drugs to normal tissues, leading to potential side effects. For example, although endostatin has shown significant therapeutic effects in lung cancer, ovarian cancer and lymphoma, it is easily distributed in all tissues, and more than 90% -95% of endostatin is accumulated in normal tissues and organs (Guo et al., 2019; Y. Chen et al., 2011; Lopes-Coelho et al., 2021). This endostatin accumulated in the heart can cause cardiomyocyte apoptosis and mitochondrial dysfunction through the transcription factor p53 (Guan et al., 2023). In clinical practice, about 6.38% of patients may have different degrees of adverse cardiac reactions, especially about 2.1% of patients have to terminate treatment due to cardiac reactions (Anakha et al., 2023). In nano therapy, whether it is based on the endogenous reactivity of tumour microenvironment pH (Wang et al., 2020), glutathione (GSH) (Lyu et al., 2020), reactive oxygen species (ROS) (Chen et al., 2021)), or the exogenous reactivity based on magnetic field (Du et al., 2020) and phototherapy (Chang et al., 2021; Zeng et al., 2021), nano enzymes have shown good therapeutic potential. However, while the enhanced permeability and retention (EPR) effect can effectively promote the accumulation of nanoenzymes at tumour sites, this enrichment still accounts for less than 1% of the total nanoparticles (Lammers et al., 2016; Torrice, 2016). At present, a variety of targeted modification strategies are being explored to enhance the tumour-targeting ability of nanoenzymes. However, the complex physiology of the human body poses a major challenge for the effective delivery of nanoparticles to tumour sites. In contrast, these nanoparticles are often encapsulated in normal tissues, where they are often engulfed by macrophages in the

reticuloendothelial system. Therefore, they tend to accumulate in organs such as liver and spleen, resulting in prolonged exposure time and direct interaction with normal tissues. This continuous interaction will inevitably lead to long-term damage, fibrosis, and even cancer (Figure 1.2) (Yu & Zheng, 2015; Yang et al., 2019). Therefore, it is crucial to overcome the biological barrier during the process of systemic administration.

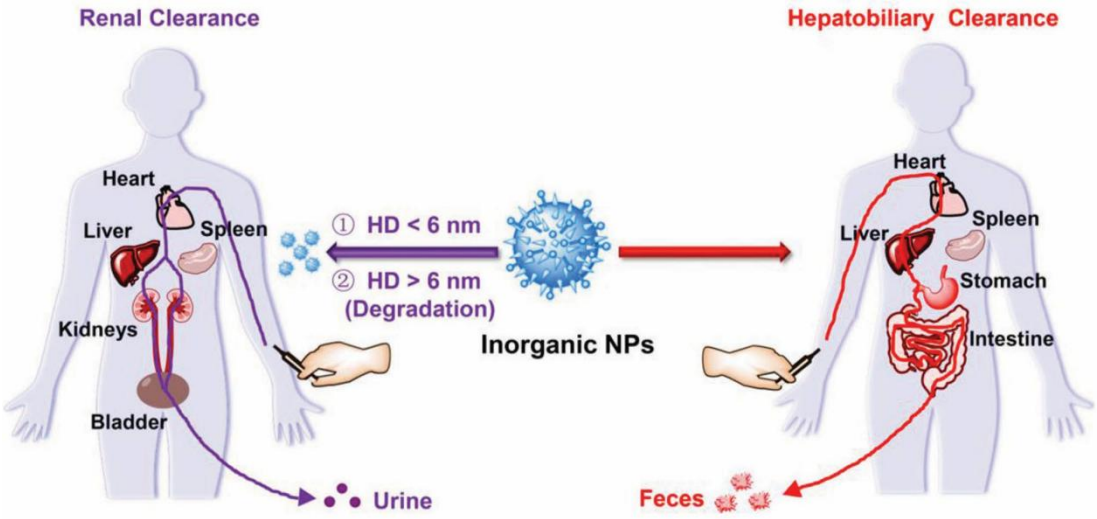

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Figure 1.2 Schematic illustration for the primary metabolic pathway of inorganic nanoparticles (NPs) in the body. NPs have two main clearance pathways in the body: renal clearance and hepatobiliary clearance. When the hydrated diameter (HD) of nanoparticles is less than 6 nm, they can be filtered by the kidneys and excreted with urine; Particles larger than 6 nm need to be degraded first and then excreted through the renal system. The renal clearance pathway involves organs such as the heart, liver, spleen, kidneys, and bladder. On the other hand, larger nanoparticles can also be metabolized through the hepatobiliary pathway, circulating through the liver, spleen, stomach, and intestines, and ultimately excreted with feces.NPs: Nanoparticles (Yang et al., 2019).

The use of traditional biological carriers such as bacteria (Zheng et al., 2022), cell membranes (Gao et al., 2022), and cell (Gao et al., 2016) is considered an effective means to address off-target issues caused by the isolation effect of

biological barriers. Among these biological delivery systems, the superior biocompatibility, low immunogenicity, no ethical controversy, and homing effector of mesenchymal stem cells (MSC) have received considerable attention (Gu et al., 2015; Zheng et al., 2022). Compared to bacteria and viruses, MSC has better biosafety and lower immunogenicity (Huang et al., 2022; Malicev & Jazbec, 2024). Compared to cell membrane encapsulation, MSC have the ability to homing into tumour tissue (O'Rourke & Kempf, 2019). It is widely believed that mesenchymal stem cells (MSC) can recognize cytokines such as TGF-β1, IL-6, CXCL12, and CXCL16 released from tumour tissue through chemokine receptors on their cell membrane surface, allowing them to home in on the tumour lesion (Table 1.1). However, cytokines such as TGF-β1, IL-6, CXCL12, and CXCL16 are not exclusively found in the tumour microenvironment. For example, TGF-β1 is highly expressed in systemic sclerosis, IL-6 in inflammatory bowel disease, and CXCL12 and CXCL16 in rheumatoid arthritis (Sasportas et al., 2009; Huang et al., 2022). Therefore, natural MSC delivery platforms may not effectively distinguish the sources of these chemokines, potentially leading to off-target effects.

Table 1.1 The cytokine/receptor pairs involved in the active tumour targeting of MSC

Tumour Type	Tumour Cell Line	Tumour Tissue Inflammatory Factors	MSC Source	Corresponding Receptors on MSC
Breast Cancer	SUM1315, MDA-MB-231	TGF-β1	Human bone marrow	—
	4T1	TGF-β1, Vascular Endothelial Growth Factor, Platelet-derived Growth Factor BB	Mouse bone marrow	CCR2

Liver Cancer	MDA-MB-231, MCF-7, MDA-MB-468	IL-6	Human bone marrow	—
	SNU-398, Hep3B	CXCL12	Human bone marrow	CXCR4
	HuH7	Macrophage Inflammatory Protein 1α, Macrophage Inflammatory Protein 3α, Matrix Metalloproteinase 1	Human bone marrow	—
	HCCLM3	Vascular Endothelial Growth Factor, CXCL9, CCL25, Matrix Metalloproteinase 9	Mouse bone marrow	—
Glioma	U251	CXCL12	Human umbilical cord blood	CXCR4
	U87	IL-8	Human umbilical cord blood	CXCR1
	U87, U251, LN229	Platelet-derived Growth Factor BB, Vascular Endothelial Growth Factor, CXCL12	Human bone marrow	—
Osteosarcoma	Saos-2	CXCL12	Human bone marrow	CXCR4
Medulloblastoma	Daoy, D283	CXCL12	Human umbilical cord blood	CXCR4

To address the off-target effects of natural MSC delivery platforms, modification is essential. In recent years, advancements in synthetic biology have enabled the construction of artificial sensing and response pathways in cells. These pathways allow cells to recognize specific environments and trigger customized responses accordingly. Notch protein is an evolutionarily highly conserved cell membrane surface receptor, which can regulate the development of biological cells by recognizing notch ligand (DSL protein) (Nowell & Radtke, 2017). Previous studies have shown that Notch protein can still perform notch-like functions by

retaining only the smallest transmembrane core domain even though replacing intracellular and extracellular domains (Gordon et al., 2015; Morsut et al., 2016).

Her2 is a protein encoded by the Her2 gene that participates in the regulation of cell growth and division. It is closely related to the occurrence and progression of tumours and is a common tumour marker and target. Her2 (+) tumours account for 15-20% of all breast cancer cases (Exman et al., 2009), and are also found in gastric cancer, ovarian cancer, bladder cancer and other cancers (Zhu et al., 2024). Compared with Her2 (-) tumours, Her2 (+) tumours have the characteristics of high invasiveness and poor prognosis (Exman et al., 2009). The differentiation between Her2 (+) and Her2 (-) tumours holds significant clinical importance and is commonly evaluated through a combination of immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). IHC is primarily used to detect Her2 protein expression on the cell membrane of tumour cells, with semi-quantitative scoring categorized as 0, 1+, 2+, and 3+. Scores of 0 and 1+ indicate Her2 (-), while a score of 3+ signifies Her2 (+). A score of 2+ is considered equivocal and requires further analysis with FISH. FISH employs fluorescently labeled probes to assess Her2 gene amplification. When the Her2/CEP17 ratio is ≥2.0, Her2 gene amplification is confirmed, and the tumour is classified as Her2 (+); if the ratio is <2.0, it is considered Her2 (-) (Hofheinz et al., 2023). The combined use of IHC and FISH allows for a more comprehensive evaluation of Her2 status, assisting clinicians in determining the need for Her2-targeted therapy(Sasi et al., 2009). Given the substitutability of the modular structure of Notch protein, this study replaced the extracellular and intracellular domains of Notch protein with Her2-specific antigen receptor (affibody) and VP64, respectively, to construct a notch-like intelligent artificial signaling pathway that can recognize Her2 (+) tumours.

Due to their high expression of the Her2 protein, Her2 (+) tumours differ significantly from normal tissues. Intelligent drug delivery platforms equipped with an artificial signalling pathway (Affibody-Notch (core)-VP64-GAL4/UAS-HSV-TK) can enable mesenchymal stem cells (MSC) to selectively identify Her2-positive tumours by recognizing the Her2 protein on tumour cells. When the recognition structure of these platforms detects the Her2 protein on tumour cells, VP64 transmits a signal that activates the GAL4/UAS gene expression regulatory element, specifically initiating the expression of a drug release gene in the response structure. Conversely, in normal tissues with low expression of Her2 protein, the intelligent drug delivery platforms recognize these cells as normal tissue and do not activate the coupled artificial signalling pathway or the drug release gene expression. This intelligent recognition mechanism enables the precise release of drugs at tumour sites while effectively preventing drug release in normal tissues, leveraging the unique artificial signalling pathway of the intelligent drug delivery platforms. Since drugs carried by cells can generally be divided into exogenous drugs and protein drugs that can be expressed by cells themselves (Li et al., 2020; Zhang et al., 2022), to enable intelligent drug delivery platforms to carry these two types of drugs, this study designed two types of drug delivery systems: the rupture release drug mode ($MSC^{intelligent}$) and the secretion drug mode ($iMSC^{Endostatin}$). Both of these intelligent drug delivery platforms can autonomously distinguish between tumour and non-tumour tissues, selectively releasing drugs only at the tumour site. Therefore, these platforms represent a new paradigm in tumour-targeted therapy.

1.2 Problem Statement

Globally, tumours account for approximately 9.7 million deaths each year, highlighting an urgent need for therapies that can target tumours specifically while

minimizing harm to healthy tissues (Bray et al., 2024). Particularly, Her2-positive tumours present a significant challenge due to their poor prognosis, tendency for metastasis, and high recurrence rates (Xie et al., 2022). Although traditional targeted therapies like antibody-drug conjugates (ADCs) and cell carriers show promise in treating Her2-positive tumours (Dean et al., 2021; Wang et al., 2022), biological barriers still hinder drugs from reaching the tumor site, thereby causing damage to normal cells. For instance, while ADCs enhance drug affinity towards tumours, they struggle with chemotaxis to tumour sites and overcoming biological barriers (Thanuja et al., 2018).

Cell vectors, which recognize tumour-released cytokines via surface chemokine receptors, can deliver drugs to tumour sites (Ali et al., 2022; Gao et al., 2022). However, diseases like viral myocarditis share similar microenvironmental characteristics with tumours, complicating accurate targeting (Zhu et al., 2022; Zhu et al., 2022). Moreover, the inherent receptors on cell carrier surfaces are not primarily designed to target tumour microenvironments, posing challenges in effectively distinguishing chemokine sources and potentially leading to off-target delivery or loss of direction. Current targeting strategies focus on enhancing drug accumulation in lesions yet addressing off-target effects—where drugs inadvertently affect healthy tissues outside biological barriers—is equally critical. However, research has predominantly concentrated on increasing drug concentrations within tumours, neglecting strategies to prevent drug presence in healthy tissues.

Therefore, this study aims to develop a drug delivery platform capable of accurately distinguishing Her2-positive tumours from healthy tissue cells and selectively releasing drugs specifically at the tumour site. This approach aims to

minimize drug presence in healthy tissues, thereby alleviating toxic side effects associated with traditional therapies.

1.3 Objectives of This Study

1.3.1 General objective

The purpose of this study was to develop a MSC-derived artificial signal pathway delivery platform for the targeted therapy of Her2 (+) tumours.

1.3.2 Specific objectives

(1) To develop an intelligent drug delivery platform that can autonomously recognize the tumour and ruptured drug release mode by endowing MSC with Affibody-Notch (core)-VP64-GAL4/UAS-HSV-TK artificial signalling pathways ($MSC^{intelligent}$).

(2) To evaluate the enhanced functionality of $MSC^{intelligent}$ that can recognize the Her2 (+) tumour and secrete drug by modifying the MSC with Affibody-Notch (core)-VP64-GAL4 expressing endostatin (known as Affibody-Notch (core)-VP64-GAL4/UAS-Endostatin) artificial signalling pathways ($iMSC^{Endostatin}$).

(3) To evaluate the recognition efficiency of Her2 (+) tumours and the anti-tumour effects of intelligent drug delivery platforms using both in vitro and in vivo model systems.

CHAPTER 2

LITERATURE REVIEW

The epidermal growth factor receptor (EGFR) family (Her family) plays a crucial role in cell growth, development, and differentiation. Overexpression of the epidermal growth factor receptor can lead to disruption of normal cellular functions and is often closely associated with the occurrence and progression of tumours. Among the family members, human epidermal growth factor receptor 2 (Her2) overexpression is closely related to the degree of malignancy in many epithelial cell cancers. Tumours with high Her2 expression exhibit strong migratory and invasive characteristics, poor sensitivity to chemotherapy drugs, poor prognosis, and a tendency for recurrence (Harari & Yarden, 2000). This scenario significantly impacts patients' quality of life and the effectiveness of their treatment.

2.1 The Structure and function of Her2

Her2 belongs to the human epidermal growth factor receptor family, which consists of four members: Her1, Her2, Her3, and Her4, as shown in Table 2.1. All these receptors are located on the cell membrane surface and share comparable structures, featuring an extracellular segment binding to ligands, a single transmembrane α, a spiral transmembrane section, and an intracellular region comprising the near membrane area, the tyrosine kinase catalytic domain, and the C-terminal tail region, which is abundant in tyrosine for regulatory purposes (Oh & Bang, 2020). The extracellular domain can be further divided into four subdomains I, II, III, and IV regions (Figure 2.1). Leucine-rich domains I and III participate in ligand binding, while cysteine-rich domains II and IV participate in receptor dimerization activation (Bai et al., 2023). The activation process of other member

receptors involves a series of receptor structural changes and phosphorylation processes, including ligand-induced receptor extracellular domain conformations, extracellular domain dimerization, a transmembrane domain, intracellular near membrane domain, tyrosine kinase domain conformations, C-terminal phosphorylation, and others (Friedlaender et al., 2022).

Table 2.1 Epidermal growth factor receptor family

Gene Encoded Product	Molecular Weight (kDa)	Tyrosine Kinase	Antagonists
EGFR	170	+	EGF, TGFα, AR, BTC, EPR, HB-EGF
HER2/neu	185	+	–
HER3	160	–	HRG
HER4	180	+	BTC, EPR, HB-EGF, NRG-2, NRG-3

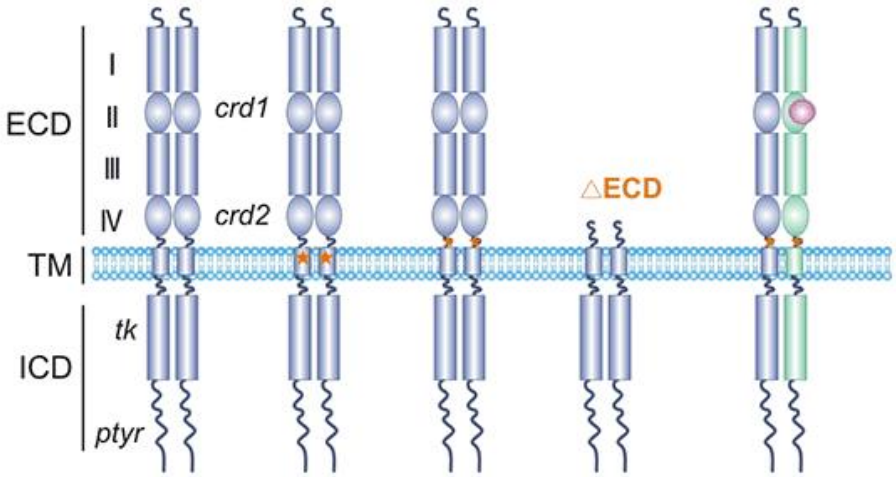

Figure 2.1 Her2 Structure. The Her2 receptor family has a similar domain organization: the extracellular domain (ECD) contains two cysteine rich domains (CRDs); Transmembrane domain (TM); And intracellular domain (ICD), which is composed of tyrosine kinase (TK) domain and C-terminal tail, in which tyrosine can be phosphorylated (pTyr) (Adapted from Bai et al., 2023).

Nevertheless, Her2 consistently remains activated and lacks soluble specific ligands due to its distinctive open conformation, distinguishing it from other members (Arteaga & Engelman, 2014; Niazi et al., 2018; Roskoski, 2014). As shown in Figure 2.2, Her2 forms heterodimers with Her3 and Her4 in its family, and tyrosine kinase activity in cells is activated. Thus, the plasma membrane RAS • GDP is converted into RAS • GTP in the cell membrane. Finally, mitogen-activated protein kinase (MAPK) is activated in cascade. This activation of ERK can transmit signals into the nucleus, leading to the activation of transcription for various transcription factors, such as c-myc, c-fos, and c-jun (Berrino et al., 2022). The expression level of Her2 is extremely low in normal cells, but it is highly expressed during embryonic development (Arteaga & Engelman, 2014; Roskoski, 2014), playing an important regulatory role in cell proliferation, differentiation, development, adhesion, and migration.

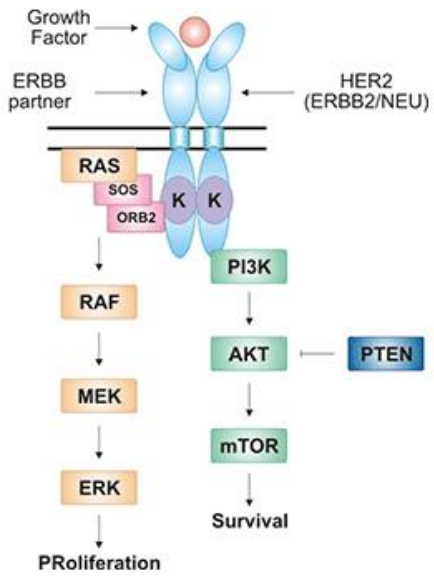

Figure 2.2 Her2 mediated signal transduction pathway. GRB2 : Growth factor receptor-bound protein 2; ERK: Extracellular signal-regulated kinase; PI3K: Phosphoinositide 3-kinase; mTOR: Mechanistic target of rapamycin; PTEN: Phosphatase and tensin homolog (Berrino et al., 2022).

The amplification or overexpression of the Her2 gene is present in various cancers such as breast, ovarian, gastrointestinal, lung, and others. Particularly in breast cancer, Her2 has been identified as both a predictor and therapeutic target (Klocker & Suppan, 2020). Another distinguishing feature of Her2 compared to other members is its limited internalization. Unlike other receptor families, which undergo ligand-dependent endocytosis processes within cells to maintain a balanced state, Her2 exhibits poor internalization (Bertelsen & Stang, 2014; Zhang & Simons, 2014). Numerous studies have shown that the Her receptor family can undergo endocytosis through various pathways, such as ubiquitin-dependent, ubiquitin-independent, and grid protein-dependent pathways, which play an important regulatory role in the signalling pathways it mediates (Bai et al., 2023). Due to its unique structure, Her2 exhibits different endocytic characteristics. Research has shown that Her2 is mainly localized on the cell surface, and receptor activation does not lead to its effective endocytosis (Berrino et al., 2022). The nature of this impaired endocytosis is influenced by the intracellular domain at the receptor's C-terminus. Additionally, it may result from the inability of internalized Her2 to effectively enter multivesicular bodies and recycle back to the membrane. However, the specific mechanism is still unclear (Bertelsen & Stang, 2014; X. Zhang & Simons, 2014; Klocker & Suppan, 2020).

2.2 The relationship between the Her2 gene and tumours

Typically, Her2 expression is limited to fetal development, and in adulthood, its low-level presence on the cell surface can only be detected in very few tissues through immunohistochemical staining. In human cancers, molecular changes in Her2 commonly entail overexpression of the normal gene product. This overexpression is noted in several human cancers, including breast cancer, ovarian

cancer, lung adenocarcinoma, and primary renal cell carcinoma (Barbar et al., 2022; Norwood et al., 2022; Takada & Toi, 2020; Vries et al., 2023). Due to the localization of Her2/neu protein in the plasma membrane, its diffusion is limited to both directions, and even moderate levels of Her2 expression can lead to activation of its receptors (Connell & Doherty, 2017).

2.2.1 Her2 gene and breast cancer

Approximately 15-20% of breast cancer cells exhibit overexpression of the Her2 gene, a phenomenon closely associated with tumour recurrence and metastasis (Fernande et al., 2023). The expression level of the Her2 gene is positively correlated with rates of tumour recurrence and metastasis (Ponde et al., 2019). Her2 has become an independent marker for assessing the prognosis of breast cancer (Ponde et al., 2019). Breast cancers with Her2 overexpression exhibit distinct biological characteristics. Among the four molecular subtypes of breast cancer, Her2-overexpressing tumours show significant invasiveness, higher metastatic potential, and greater malignancy (Prat et al., 2014). Her2 levels can serve as an independent marker for evaluating the prognosis of breast cancer. Among HR+ and Her2- breast cancer patients, the survival rate is the highest. However, at advanced stages, Her2+ patients have a better survival rate than Her2- patients (Moutafi et al., 2022). There is no consensus on the impact of Her2 gene overexpression on the efficacy of chemotherapy drugs, but most studies indicate that the gene is sensitive to anthracycline-based chemotherapy (Ramshorst et al., 2018).

2.2.2 Her2 gene and lung cancer

The mutation status of the Her2 gene varies among different types of lung cancer. Buttitta et al. analyzed Her2 gene mutations in 403 Caucasian lung cancer patients. Their findings revealed that mutations were predominantly observed in

bronchioloalveolar adenocarcinoma, with a slightly higher occurrence among females compared to males, as well as in nonsmokers compared to smokers (Buttitta et al., 2006). Studies have shown that Her2 overexpression mainly occurs in non-small cell lung cancer, predominantly adenocarcinoma rather than squamous cell carcinoma (Merkhofer et al., 2022). Lashkarizadeh et al. found that Her2 was not expressed in normal lung tissues but had a positive expression rate of 26.67% in lung cancer tissues, all of which were adenocarcinomas, with no expression observed in small cell lung cancer or squamous cell carcinoma (Lashkarizadeh et al., 2023). However, The overexpression of Her2 is unrelated to the degree of lung cancer differentiation (Loeffler et al., 2023).

2.2.3 Her2 Gene and pancreatic cancer

Recent studies have shown that the expression rate of the Her2 gene in pancreatic cancer tissues is 25-30% (Bodea et al., 2024). Although the expression of Her2 in pancreatic cancer tissues is higher than that in benign lesions and normal pancreatic tissues, there is still no consensus on whether Her2 can be used as a prognostic factor for pancreatic cancer. Some studies have indicated that low Her2 expression is a predictor of poor prognosis (Sanomachi et al., 2023). In addition, Her2 may serve as a therapeutic target for pancreatic ductal adenocarcinoma (Han et al., 2021), and some new anti-Her2 drugs have demonstrated anti-tumour effects in tumours with low expression of Her2 (Randall et al., 2024).

2.2.4 Her2 Gene and Gastric Cancer

Reports on the overexpression of the Her2 gene in gastric cancer cells vary widely, with expression rates ranging from 6% to 35% (Li et al., 2022). This variability may be related to differences in sample size, detection methods, and scoring criteria. Bozzetti et al. confirmed the expression of Her2 protein and gene

abnormalities in gastric cancer (Bozzetti et al., 2011). Zhu et al. reported that higher levels of Her2 overexpression in gastric cancer tissues are associated with deeper infiltration, increased lymph node metastasis, and increased distant metastasis (Zhu et al., 2024).

2.3 Targeted therapy of Her2 tumour

Targeted therapy represents an approach to treatment functioning at the cellular and molecular levels. It targets specific oncogenic sites using tailored therapeutic agents. These agents directly interact with the identified oncogenic sites, either inhibiting tumour growth or inducing apoptosis in tumour cells while sparing surrounding normal tissues. Compared to conventional treatments, targeted therapy is characterized by high selectivity and minimal side effects.

Antibodies directed against ligands or cytokines typically act by inhibiting their attachment to receptors. Conversely, antibodies that target surface receptors or proteins exhibit a wide range of mechanisms. Apart from hindering receptor-ligand interactions, they also encompass blocking receptor-receptor or receptor-co-receptor interactions essential for receptor activation. Moreover, they engage in binding to tumour-specific antigens to facilitate antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), resulting in the demise of tumour cells (Oh & Bang, 2020). The latter requires targeting antigens, has high tumour specificity, and is not prone to endocytosis or detachment from the surface (Escriva-de-Romani et al., 2018; Klapper et al., 2000). Her2 combines these two characteristics and is therefore very suitable as a target for antibody therapy (Escriva-de-Romani et al., 2018). Numerous studies have shown that antibody recognition epitopes directly determine their impact on the dimerization form of

Her2 (Escriva-de-Romani et al., 2018; Oh & Bang, 2020). Antibodies bound to certain epitopes act as ligands, promoting the homodimerization activation of Her2; Antibodies that bind to other epitopes can lead to Her2 endocytosis; Antibodies that bind to key dimerization sites in regions II and IV have a significant inhibitory effect on their dimerization activation (Klapper et al., 2000). The currently marketed Her2 therapeutic antibodies, trastuzumab and pertuzumab, belong to the last category.

Her2 targeted therapy is effective for breast cancer patients with Her2 overexpression and/or amplification detected by IHC or FISH. Trastuzumab was the first monoclonal antibody developed in 1990 against Her2, which interferes with Her2 signalling through various mechanisms: inhibiting dimerization, receptor internalization, and/or degradation; Inhibiting the PI3K-AKT signalling pathway; And antibody-dependent cytotoxicity (ADC) (Figure 2.3) (Oh & Bang, 2020). Initially (2001), the addition of trastuzumab to chemotherapy was found to improve the overall survival time (OS) of women with Her2-positive metastatic breast cancer (Slamon et al., 2001). Later, in 2005, the use of trastuzumab as an adjuvant treatment for women achieved positive results with early diseases (Piccart-Gebhart et al., 2005; Romond et al., 2005; Slamon et al., 2011). Therefore, trastuzumab has been the standard treatment for metastatic and early Her2-positive breast cancer for more than ten years.

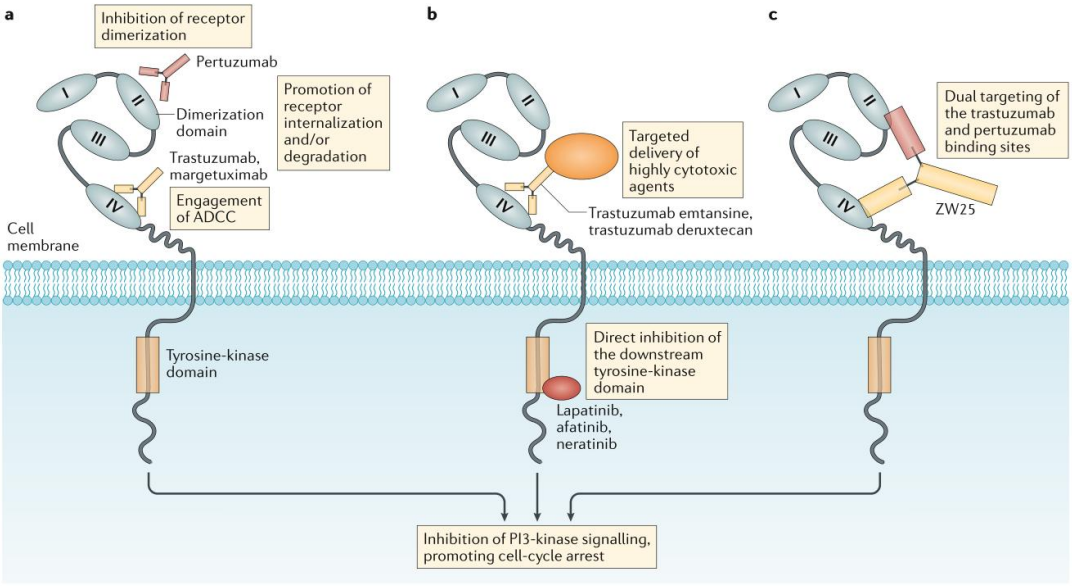

Figure 2.3 Mechanism of action of agents targeting Her2. Her2-targeted drugs achieve antitumour effects by inhibiting downstream signalling pathways, participating in antibody-dependent cellular cytotoxicity, or inhibiting receptor dimerization (Oh & Bang, 2020).

Pertuzumab is a recombinant humanized monoclonal antibody against IgG1 of Her2 protein, and its binding site is different from trastuzumab. Patuzumab binds to the dimerization domain (extracellular domain 2) of the Her2 protein, thereby inhibiting ligand-induced heterodimerization of the Her2 protein (Mann et al., 2001; Mendoza et al., 2002; Mullen et al., 2007; Sakai et al., 2007). The combination of trastuzumab and pertuzumab has been shown to synergistically inhibit tumour growth in both in vitro and in vivo preclinical models (Nahta et al., 2004; Scheuer et al., 2009). In the Phase III trial, compared to placebo with trastuzumab and docetaxel, the combination of trastuzumab and docetaxel prolonged the median OS by 15.7 months without any increase in the risk of cardiac toxicity (Baselga et al., 2012). The data from the Fujimoto study also shows that adding patuzumab to adjuvant trastuzumab plus chemotherapy can improve the invasive disease-free survival

disease-free survival (DFS) of operable Her2-positive breast cancer patients (Fujimoto et al., 2007).

Tyrosine kinase inhibitors (TKIs), such as lapatinib and neratinib, are different from trastuzumab and pertuzumab. They inhibit the growth and proliferation of tumour cells by inhibiting Her2 kinase activity and blocking downstream signal transduction. Unlike monoclonal antibodies, TKIs, as a small molecule drug, can penetrate the cell membrane and directly act on the Her2 kinase domain in cells (Zhong et al., 2024).

Lapatinib is a dual target tyrosine kinase inhibitor, which can not only inhibit HER2, but also inhibit the kinase activity of EGFR. It is usually used in patients with metastatic Her2 positive breast cancer who are resistant to trastuzumab (Bonde et al., 2018). Lapatinib combined with capecitabine can effectively control the disease progression of Her2 (+) positive breast cancer patients (Xu et al., 2021). In addition, studies have shown that lapatinib also shows certain efficacy in patients with Her2 positive brain metastasis, because it can penetrate the blood-brain barrier (Wang et al., 2023).

Neratinib is an irreversible tyrosine kinase inhibitor that can significantly inhibit the growth of Her2 tumours (Guo et al., 2023). Compared with lapatinib, naratinib can irreversibly inhibit Her2 kinase, thus providing a more lasting blocking effect (Collins et al., 2019). Naratinib is often used as adjuvant therapy for Her2 positive breast cancer, especially after initial trastuzumab treatment, to reduce the risk of cancer recurrence (Harbeck et al., 2024). Clinical studies have shown that naratinib can significantly reduce the recurrence rate of early Her2 positive breast cancer (Echavarria et al., 2017).

2.4 Limitations of traditional treatment for Her2 tumours

Her2 targeted therapy has made significant progress in improving the survival rate of Her2 (+) tumour patients, but its side effects cannot be ignored. Cardiotoxicity is one of the most common serious side effects, which may lead to heart failure and a decrease in left ventricular ejection fraction (Zhu et al., 2024). Therefore, patients receiving treatment need to undergo regular cardiac function monitoring to promptly detect and address heart-related issues. In addition, Her2 targeted therapy may also trigger a series of allergic reactions, including symptoms such as rash, fever, and chills (Ross et al., 2009). In some serious cases, it may lead to allergic shock and endanger life. This requires close monitoring of the patient's allergic reactions during the treatment process and preparing emergency response measures. Gastrointestinal reactions are also one of the common side effects of Her2 targeted therapy (Kawakami & Yamazaki, 2024). Patients often experience discomfort symptoms such as nausea, vomiting, and diarrhoea, which not only affect their quality of life but may also lead to weight loss and malnutrition. To alleviate these discomforts, medication management is usually needed to alleviate symptoms and prevent further health problems (Kawakami & Yamazaki, 2024). Fatigue is another significant side effect of Her2 targeted therapy. Patients who receive long-term treatment often feel extreme fatigue, which not only affects their daily life and work abilities but may also lead to psychological problems such as anxiety and depression (Dawood et al., 2021). Therefore, in the treatment process, management and psychological support for fatigue are also very important. In addition, Her2 targeted therapy may have an impact on the immune system and increase the risk of infection. Patients receiving treatment may be more likely to suffer from various infectious diseases, so it is necessary to strengthen protective measures and seek medical advice in time when

signs of infection appear (Fu et al., 2023). Beyond these side effects, another major challenge related to Her2 targeted therapy is drug resistance (Echavarria et al., 2017). Although these treatments show remarkable effectiveness in the early stages, drug resistance gradually emerges over time, diminishing the therapeutic effect (Zhu et al., 2024). The mechanisms leading to drug resistance are diverse and include changes in Her2 expression levels, the activation of signal pathways (such as PI3K/Akt pathway), the production of Her2 gene mutations or isomers, mutations or alterations in the Her2 gene, upregulation of multi-drug resistance genes, modifications in the tumour microenvironment, and immune evasion (Yoon et al., 2024). Overall, although Her2 targeted therapy has made significant progress in tumour treatment, its side effects have a significant impact on the quality of life of patients.

2.5 Mesenchymal stem cells

MSC are a type of adult pluripotent stem cells derived from the human mesoderm, widely present in various human tissues such as bone marrow, placenta, umbilical cord, umbilical cord blood, adipose tissue, skeletal muscle, tendons, synovium, skin, salivary glands, dental pulp, periodontal ligament, peripheral blood, menstrual blood, endometrium, amniotic fluid, and amniotic membrane (Mushahary et al., 2018; Reichert et al., 2021). MSC are a type of adherent spindle-shaped cells with high multidirectional differentiation and self-renewal potential. According to the standards of the International Society for Cell Therapy, MSC needs to have at least the following characteristics: (1) adherent growth under standard culture conditions; (2) The expression of cell surface markers such as CD73, CD90, and CD105 is positive (≥ 95% positive), while the expression of cell surface markers such as CD14, CD34, CD45 or CD11b, CD79 α or CD19, and human leukocyte antigen DR (HLA-DR) is negative (≤2% positive); (3) It can induce differentiation