Chapter 2

Literature review

2 Literature review

2.1 Skin cancer and melanoma

The skin is the largest organ in the body and protects the body from heat, direct sunlight, infections, and trauma. In addition, it stores water, fat, and vitamin D and controls body temperature. The skin is composed of three main layers: epidermis (upper or outer layer), dermis (lower or inner layer), and subcutaneous tissue (Figure 2.1). Skin cancer primarily starts at the epidermis, which is composed of three different types of cells: (i) squamous cells, which are thin, flat cells that make up the epidermis' top layer; (ii) basal cells, which are round cells that lie beneath the squamous cells; and (iii) melanocytes, which are cells that produce melanin and are located in the epidermis' lower layer. Skin becomes darker due to the increased pigment (melanin) secreted by melanocytes when exposed to artificial or solar light.

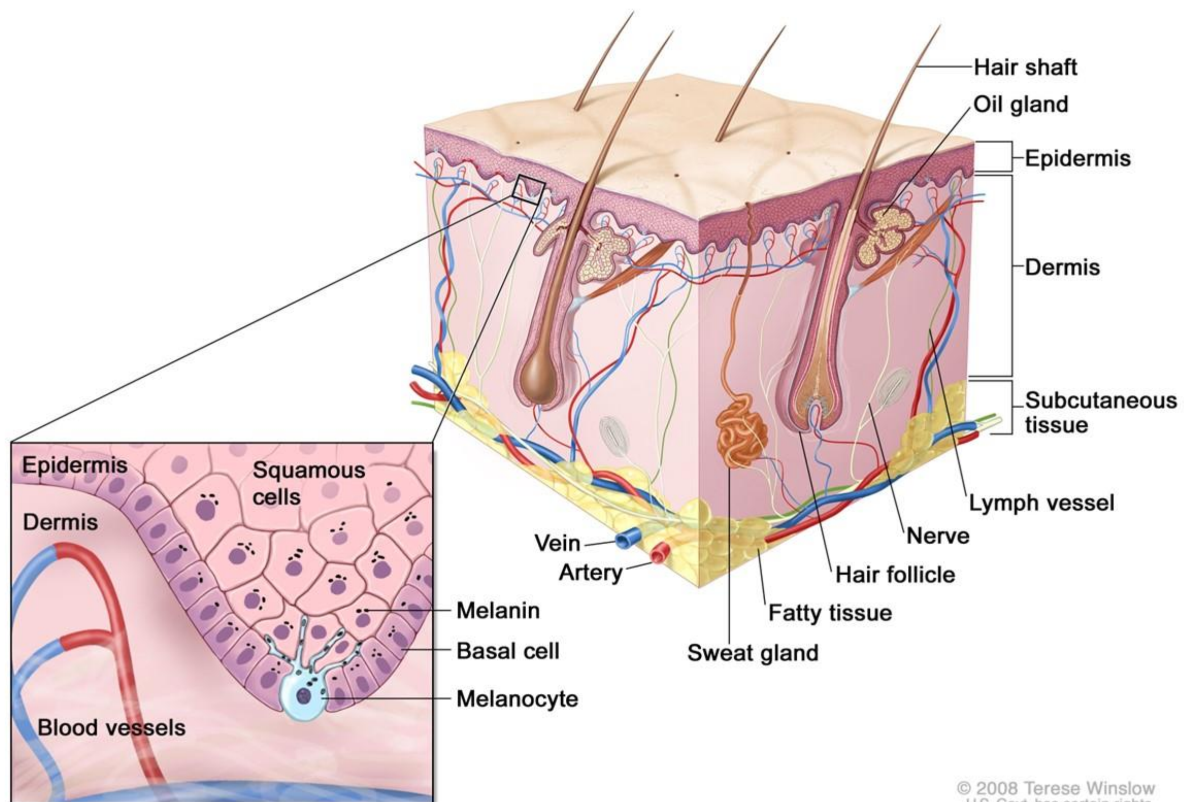

Figure 2.1 Anatomy of the skin, showing the epidermis, dermis, and subcutaneous tissue. Melanocytes are in the layer of basal cells at the deepest part of the epidermis.

Figure adapted from National Cancer Institute (NCI), USA

Skin cancer is primarily classified into three categories based on molecular profiles: basal-cell carcinoma (BCC), squamous-cell carcinoma (SCC), and melanoma [62]. Non-melanoma skin cancers, BCC and SCC, account for 80 and 16% of all skin cancer cases, respectively, while melanomas make up only 4%. BCCs are typically slow-growing and rarely metastasize, whereas SCCs can be more aggressive and have the potential to metastasize [63]. In contrast, melanoma arises from melanocytes and is associated with a high mortality rate due to its aggressive metastasis and heterogeneous nature [64].

Melanoma is the most aggressive and deadly form of skin cancer, arising from the malignant transformation of melanocytes. While more prevalent in fair-skinned Caucasian populations, melanoma can also affect individuals with pigmented skin in Asia and Africa, albeit at a lower rate. It may also manifest at a low incidence on the soles of the feet, mucous membranes, and nail beds [1, 65]. Melanoma is the fifth most prevalent cancer in the United States, with an anticipated 97,610 new cases and 7,990 deaths in 2023 (Figure 2.2), according to data from the National Cancer Institute's (NCI) epidemiology survey. Globally, there were 3,24,635 cases of melanoma cancer in 2020 for both sexes, according to data from the Global Cancer Observatory (GCO). Europe had the highest number of cases (150627), followed by Northern America (105172), Asia (23753), Oceania (19239), Latin America and the Caribbean (18881), and Africa (6963) [2].

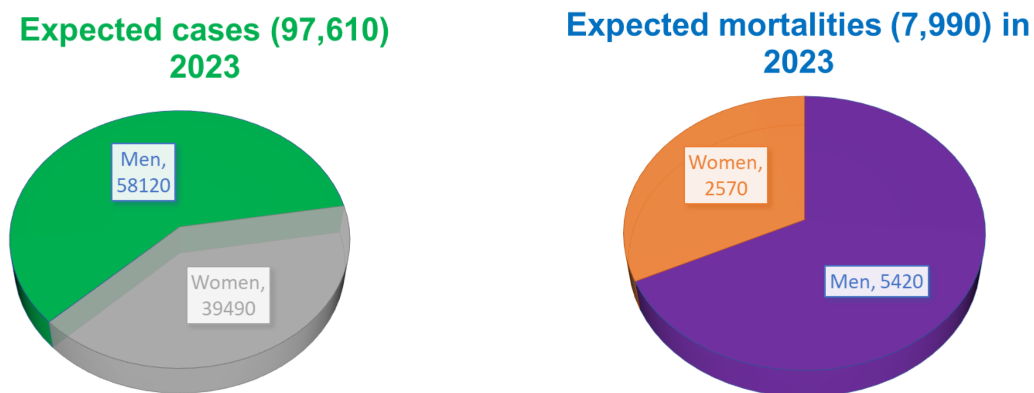

Figure 2.2 Expected cases and expected mortalities of melanoma in the US by 2023.

2.1.1 Risk factors

Melanoma is considered a complex medical condition that arises from a combination of environmental exposure and genetic factors [66]. While it is multifactorial, excessive UV radiation exposure is the primary risk factor, leading to DNA damage, inflammatory responses, and genetic alterations [67]. Other contributing factors to the development and progression of melanoma include sunburn, genetic predisposition, family history of cancer, numerous freckles, presence of dysplastic nevi, reduced DNA repair capacity, inability to tan, aging, compromised immune system, mutations in cyclin-dependent kinase inhibitor 2A (CDKN2A or p16) and cyclin-dependent kinase 4 (CDK4) [6, 68]. The chance of developing melanoma is increased by about 50% by certain phenotypic traits, including fair skin, red hair, light eyes, lots of freckles, UV sensitivity, and an inability to tan [69].

2.1.2 Diagnosis

Various methods are available to detect the early and later stages of melanoma they are (i) skin self-examination, (ii) dermoscopy, and (iii) total-body photographic images and short-term surveillance. Self-examination of the skin has great potential as a rapid and simple method of screening for melanoma and precancerous lesions [70]. To diagnose melanoma, the "ABCD" criteria were created in 1985 (Figure 2.3). "Asymmetry," "Border irregularity," "Color variegation," and "Diameter >6 mm" are the four terms that make up the acronym "ABCD" [71]. Later on, the letter "E" was added to stand for "Evolving," which is particularly crucial when diagnosing nodular melanomas. These criteria serve as a straightforward tool to help the general public and non-dermatologists distinguish between common moles and cutaneous lesions that are most likely to be early melanoma [72]. Other clinical methods have been developed to enhance early diagnosis, such as the "Glasgow 7-point checklist," which comprises four minor criteria (sensory change, diameter of 7 mm or greater, and the presence

of inflammation, crusting, or bleeding) and three major criteria (change in size, shape, and color) [6].

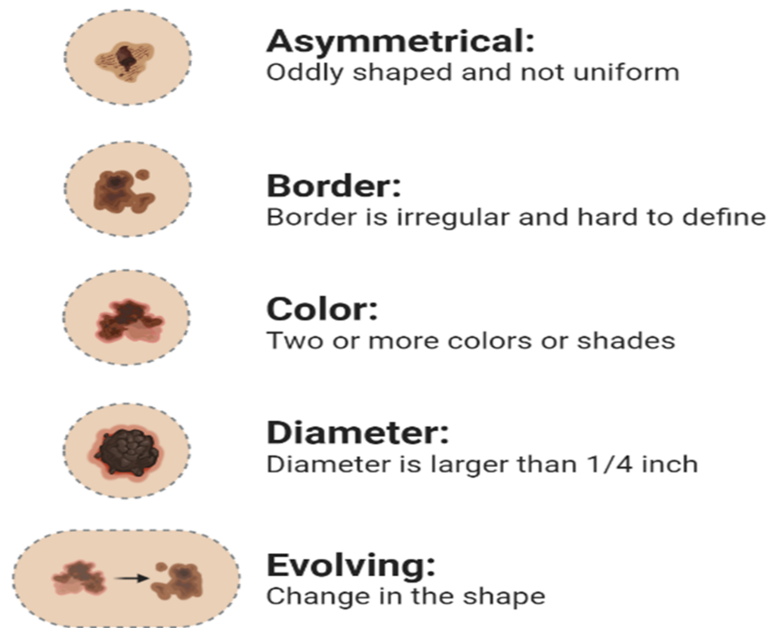

Figure 2.3 Graphical representation of melanomas with characteristic asymmetry, border irregularity, color variation, and large diameter (ABCD).

A non-invasive diagnostic method called dermoscopy uses a variety of assistive optical instruments to view morphological structures to diagnose melanoma. Both visually and dermoscopically, certain melanomas are challenging to diagnose [73, 74]. However, photographs that can be electronically taken, stored, retrieved, and examined can be created. It has been demonstrated that reflectance confocal microscopy is an effective imaging technique for identifying malignant melanocytic lesions.

2.1.3 Progression of melanoma

Melanoma progresses through several stages, beginning with the formation of acquired nevi due to increased melanocyte proliferation. This is followed by abnormal melanocyte differentiation, leading to the development of dysplastic nevi [75]. The disease then enters a radial growth phase, where a primary tumor forms within the epidermis. As melanoma advances, it transitions into a vertical growth phase, during which the tumor invades deeper into the dermis [76]. In its most advanced stage, melanoma spreads, forming metastatic lesions

in distant visceral organs, which results in malignancy and significantly increases mortality risk. Based on the tumor's thickness, its ability to spread to lymph nodes or other parts of the body, and other criteria, melanoma is categorized into several stages (Stage 0, Stage I, Stage II, Stage III, and Stage IV) (Figure 2.4) [77].

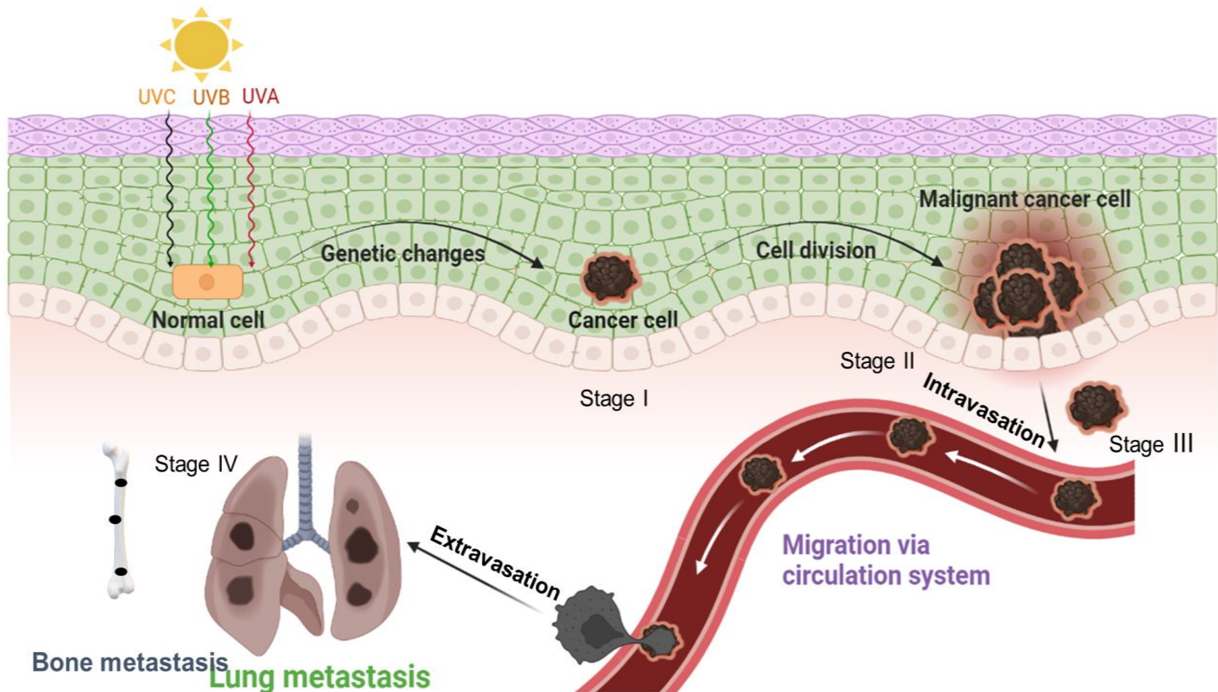

Figure 2.4 Graphical representation of the progression of melanoma.

2.1.4 Types of melanomas

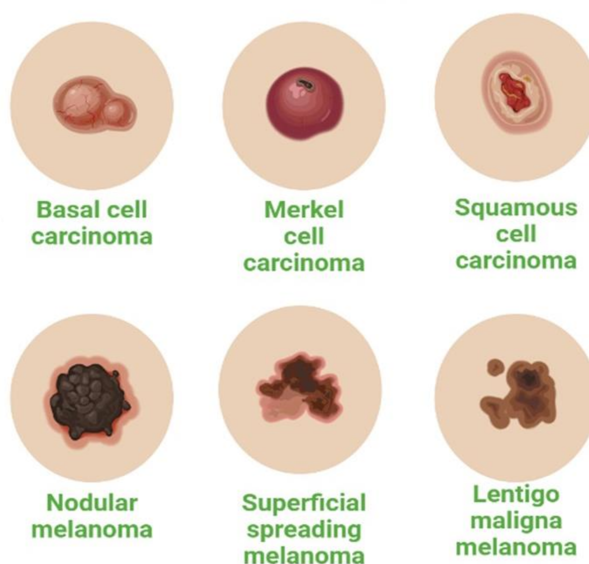

Figure 2.5 Graphical representation of types of melanomas.

Melanoma is typically categorized into two main types: mucosal melanoma and cutaneous melanoma. Cutaneous melanoma can be further classified based on clinical and histological features into nodular melanoma, lentigo malignant melanoma, acral lentiginous melanoma, superficial spreading melanoma, and desmoplastic melanoma (Figure 2.5) [1]. Other melanoma variations have been identified, such as rhabdoid, anorectal, vulvovaginal, myxoid, balloon cell, head and neck, and osteogenic melanoma [78].

2.1.5 Pathogenesis

Prolonged exposure to UV radiation can lead to melanoma, DNA damage, and genetic changes [1, 6]. The development and progression of melanoma can be influenced by mutations in CDKN2A or p16 and CDK4 [6]. Melanoma occurs when critical signal transduction pathways, such as phosphatidylinositol-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK), are overstimulated due to mutations in the BRAF and NRAS genes [10]. Both the MAPK (Ras/Raf/MEK/ERK) and PI3K/Akt pathways are activated downstream when oncogenic NRAS is triggered [79].

The MAPK pathway is a signal transduction cascade that connects various growth factors to plasma membrane growth factor receptors, such as fibroblast growth factor receptor (FGFR), epidermal growth factor receptor (EGFR), and platelet-derived growth factor receptors (PDGFR) [79]. It achieves this by transmitting extracellular signals to the nucleus through a series of consecutive phosphorylation events. The proto-oncogene RAS is activated by converting inactive RAS-guanosine diphosphate (RAS-GDP) into active RAS-guanosine triphosphate (RAS-GTP) upon receiving growth stimuli from plasma membrane receptor tyrosine kinase (RTK) [80]. The RAF protein is translocated from the cytosolic region to the cell membrane by the active form of RAS, where it is phosphorylated to become activated [81]. Activated RAF then phosphorylates MAP kinase extracellular signal-regulated kinases 1 and 2 (MEK1/2), which in turn phosphorylate specific tyrosine and threonine residues to activate

extracellular signal-regulated kinases 1 and 2 (ERK1/2) [11, 81]. The final ERK in the MAPK pathway translocates to the nucleus and phosphorylates various nuclear transcription factors, such as CREB, Elk-1, Myc, and Fos, among others, thereby promoting the survival, differentiation, and proliferation of numerous cell types. Mutations in the BRAF (50–70%) and NRAS (15–30%) genes lead to abnormal activation of the MAPK cascade and hyperphosphorylation of extracellular signal-regulated kinases (ERK) in most melanoma cases [11]. The BRAF mutation, known as Val600Glu or B-RafV600E, occurs when glutamic acid replaces valine at codon 600 in exon 15. This mutation is relatively common in cutaneous melanoma cases but rare in conjunctival, mucosal, and uveal melanomas [82]. BRAF, also known as the proto-oncogene B-RAF, is a serine/threonine protein kinase. The RAF kinase family consists of B-RAF, A-RAF, and C-RAF (also known as RAF-1). However, the BRAF mutation is uncommon in mucosal, acral, conjunctival, and uveal melanomas [79]. It's noteworthy that BRAF and NRAS mutations in melanoma rarely coexist, indicating that the activation of the MAPK pathway can be driven by either a BRAF or NRAS mutation alone [11].

The PI3K enzyme phosphorylates phosphatidylinositol-4, 5-biphosphate (PIP2) to phosphatidylinositol-3, 4, 5-triphosphate (PIP3) in response to active RTK receptors. PIP3 then activates Akt (Ak strain transforming kinase), which in turn phosphorylates and activates key downstream effector proteins of the PI3K pathway, supporting cell survival and proliferation [83, 84]. The lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) negatively regulates this cascade by dephosphorylating PIP3 [11]. When this pathway is deregulated due to PTEN loss or oncogenic RAS, it can lead to the development of melanoma. Studies have shown that Akt levels are elevated in approximately 70% of cutaneous melanomas compared to healthy melanocytes (Figure 2.6). The Akt family, including Akt, Akt 2, and Akt 3, plays a significant role as downstream effectors in transmitting signal transduction

cascades initiated by PI3K. Notably, Akt 3 is a key factor in the development of melanoma [10]. Previous studies have indicated that around 70% of cutaneous melanomas exhibit higher Akt expression levels than normal melanocytes.

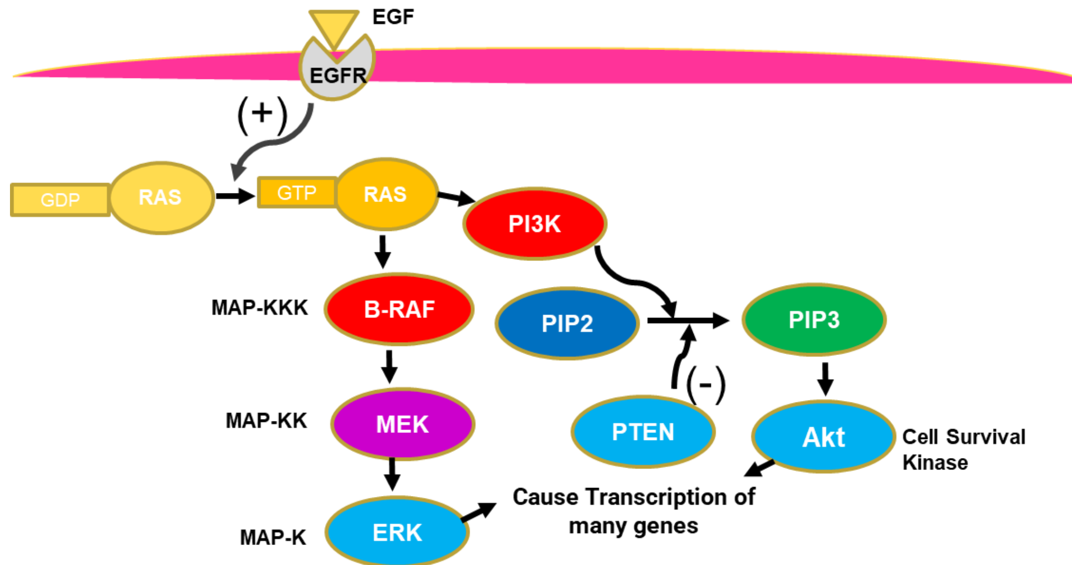

Figure 2.6 Mitogen-activated protein kinase (MAPK pathway) and phosphoinositide-3-OH kinase (PI3K/Akt).

Moreover, the development of melanoma is also influenced by immune evasion and immunosuppression. Tumor-infiltrating T lymphocytes (TILs) are important effector cells capable of recognizing and destroying tumor cells. Programmed Death Ligand-1 (PD-L1) expression can be upregulated in melanoma cells. PD-L1 exclusively binds to PD-1 receptors on the surface of TILs, leading to further suppression of their effector function through the PD-1/PD-L1 interaction.

2.1.6 Current therapeutic options for melanoma

Patients with melanoma have access to a range of treatments, including standard options currently in use and those being investigated in clinical trials. The five main types of standard treatments are (I) surgery, (II) chemotherapy, (III) radiation therapy, (IV) immunotherapy, and (V) targeted therapy. Clinical trials are exploring novel treatment approaches such as vaccination therapy [85].

Chemotherapeutic agents are utilized in chemotherapy to either kill or inhibit the division of cancer cells. The specific chemotherapy regimen used is determined by the type and stage of the melanoma being treated [70]. Dacarbazine, interleukin-2 (IL-2), and interferon-alpha (IFN-α) have been approved by the Food and Drug Administration (FDA) as chemotherapeutic agents for treating melanoma [86]. Dacarbazine has been the preferred first-line treatment for melanoma since its approval by the US FDA in 1976 [87]. IFN-α is employed as adjuvant immunotherapy for advanced melanoma, and high-dose IL-2 was granted authorization for the treatment of melanoma in 1998 [88]. Immunotherapy is an effective treatment for melanoma that harnesses the body's immune system. By strengthening, guiding, or repairing the body's natural defenses, immunotherapy helps combat melanoma. Immune checkpoint inhibitors play a crucial role in this process by blocking certain proteins, known as checkpoints, that are produced by cancer cells and immune cell types (T cells) [89]. T lymphocytes are better equipped to fight cancer cells when these checkpoints are blocked. Some patients with advanced melanoma or unresectable malignancies respond well to this treatment [90]. There are two main forms of immune checkpoint inhibitor therapy: cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) inhibitor therapy and programmed cell death protein 1 (PD-1) and programmed cell death ligand 1 (PD-L1) inhibitor therapy [85, 91]. One type of CTLA-4 inhibitor is ipilimumab (Figure 2.7). In the treatment of melanoma, researchers are studying the use of atezolizumab, a PD-L1 inhibitor, in combination with cobimetinib and vemurafenib [92]. Melanoma is often treated with other immunotherapies, including tumor necrosis factor (TNF) and interleukin-2 (IL-2). IL-2 has been found to enhance the development and function of immune cells, particularly lymphocytes, which can target and eliminate cancer cells. White blood cells release the TNF protein in response to an antigen or infection. There have been reports of using TNF for the treatment of melanoma [93].

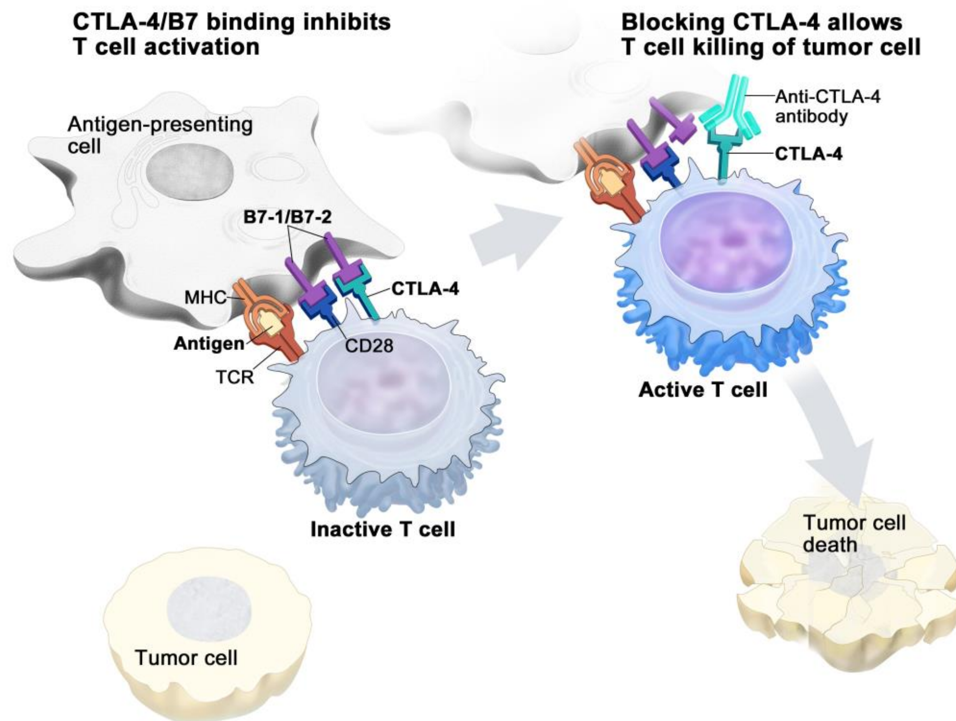

Figure 2.7 Immune checkpoint inhibitor. Checkpoint proteins, such as B7-1/B7-2 on antigen-presenting cells (APC) and CTLA-4 on T cells, help keep the body's immune responses in check. When the T-cell receptor (TCR) binds to the antigen and major histocompatibility complex (MHC) proteins on the APC and CD28 binds to B7-1/B7-2 on the APC, the T cell can be activated.

Figure adapted from National Cancer Institute (NCI), USA

Targeted therapies, such as oncolytic viral therapy and signal transduction inhibitor therapy, are known for causing less harm to normal cells compared to radiation or chemotherapy [94]. Signal transduction inhibitors play a crucial role in preventing signals from moving between molecules inside cells, which is essential for cell survival, proliferation, and reproduction [95]. Blocking these signals can lead to the death of cancer cells. BRAF inhibitors like dabrafenib, vemurafenib, and encorafenib target proteins produced by BRAF gene mutations, while MEK inhibitors such as trametinib, cobimetinib, and binimetinib block proteins known as MEK1 and MEK2, affecting the development and survival of cancer cells [96]. Oncolytic virus therapy involves targeting and eliminating cancer cells while minimizing damage to healthy cells [97]. This treatment may be followed by chemotherapy and radiation therapy. One type of oncolytic

virus therapy, known as "talimogene laherparepvec," is created using a modified strain of the herpesvirus in a laboratory. It is directly injected into the skin and lymph nodes where the tumor is located during treatment [98].

Clinical trials are underway to evaluate innovative treatments, including vaccination therapy. Vaccine therapy aims to activate the immune system to identify and eradicate tumors using specific drugs or combinations of substances. For instance, a study has examined the combined use of IL-2 and a melanoma peptide antigen vaccination (gp100) [70].

2.1.7 Various *in vivo* tumor models with special reference to the syngeneic tumor model

Various pre-clinical melanoma models have been developed to enhance our understanding of tumor biology, aiding in the diagnosis, treatment, and prevention of melanoma. These models accurately reflect the microenvironment of melanoma. Genetically engineered models (GEM), xenograft, and syngeneic models are among the most widely used pre-clinical mouse models, providing valuable insights into the progression of melanoma [99, 100].

Xenograft models are classified into two main types: patient-derived tumor xenografts (PDTXs) and cell-line xenografts. In cell line xenograft models, human melanoma cells (e.g., WM164 and WM793B) are introduced into immune-deficient mouse models, such as severe combined immune-deficient (SCID/SCID) mice or nude athymic (nu/nu) mice lacking T-lymphocytes. The PDTXs model involves implanting patient-derived tumors into immunocompromised mice, such as NOD/SCID IL-2 receptor gamma chain knockout mice or athymic nude mice [100, 101].

Melanoma cells are introduced into syngeneic models of the same species and genetic background. The B16 cell line, derived from C57BL/6J mice induced by specific chemical carcinogens, is among the most commonly used cell lines (61). Sub-clones B16F1 and B16F10, generated through *in vivo* passaging of B16 cells, are well-known. This model offers rapid tumor growth and development, allowing melanoma cells to interact with immunocompetent

T and B lymphocytes present in the human melanoma environment. It provides valuable insights for immunology research and treatment strategies. However, it's important to note that murine cell lines' growth factors and adhesion proteins differ significantly from those of human melanoma [102].

Genetically engineered models (GEMs) are based on transgenic mice with altered gene expression specific to melanoma development. Examples of GEMs include models of CDKN2A, RAS (rat sarcoma virus), PTEN/BRAF, and RET (receptor tyrosine kinase) [99, 103].

Melanoma is predominantly caused by UV radiation, and this is mimicked in GEMs, similar to how UV light naturally induces melanoma in humans. Chemicals such as 12-O-Tetradecanoylphorbol-13-acetate (TPA) and 7,12-Dimethylbenz[a]anthracene (DMBA) can be topically applied to induce skin inflammation and black spots, which eventually progress into melanoma. These chemicals are typically used in conjunction with UV and other genetically modified models to study melanoma development [100, 104].

2.2 Role of drug repurposing and flavonoids in melanoma

2.2.1 Drug repurposing

The conventional process of drug development encompasses preclinical research followed by clinical trials [105]. Preclinical investigations assess the efficacy, toxicity, pharmacokinetics, and pharmacodynamics of compounds using human tumor cell lines and animal models. Upon establishing a drug's therapeutic efficacy, it advances to the clinical trial phase, which includes Phase I, II, and III studies to evaluate its safety and effectiveness in humans. Consequently, the timeline for bringing a new drug to clinical approval typically spans 10 to 15 years and incurs costs ranging from $1 to $2 billion. Despite such significant investments, fewer than 1% of compounds are expected to progress to clinical trials, and even fewer will reach the market [26, 106].

In contrast, drug repurposing strategies focus on identifying new therapeutic uses for existing medications that have already received regulatory approval.

Drug	Other uses	Study model	Mechanism of action	Reference
Dihydroartemisinin	Anti-Malaria	*In vitro* in B16F10 melanoma	ROS Generation, Apoptosis NF-κB-DNA binding activity	[29]
Doxycycline	Anti-biotic and anti-malarial	Human (A2058 and A375) and mouse (B16F10) melanoma cells	Inhibition of the MMP-2 and MMP-9 metalloproteinases activity, activation of apoptosis signal-regulated kinase 1, c-Jun N-terminal kinase, and caspases, which induces apoptosis	[107]
Telmisartan	antihypertensive drug	Human melanoma cells A375, 518a2, and HTB140	Induction of apoptosis, generation of reactive oxygen species, and alteration of cell bioenergetics	[108]
Niclosamide	Anti-helminthic drug	*In vitro*: human and mouse melanoma cell lines. *In vivo*: a mouse xenograft model of A375 cell line	Induces cell apoptosis *via* the mitochondrial-mediated apoptotic pathway, and also inhibits tumor growth by decreasing the expression of p-STAT3, MMP-2, and MMP-9	[109]
Naproxen	Anti-inflammatory	Mice irradiated with UVB	Reduction in the incidence of tumor lesions by naproxen may be due to its ability to increase TNF-a levels and decrease PGE2.	[110]
Metformin	Anti-diabetic	Human melanoma cell lines	Induces cell cycle arrest in the G0-G1 phase, and it´s responsible for autophagy and apoptosis induction	[111]

This approach has gained traction as drugs initially sanctioned for one indication are subsequently investigated and employed to treat other medical conditions. A comprehensive review highlights the innovative strategies and potential transformative effects of drug repurposing within oncology (Table 2.1) [112].

Drug repurposing offers several intrinsic advantages, including reduced development time and cost, owing to the pre-existing knowledge regarding the safety, dosage, and toxicity profiles of established drugs. Interest in this strategy has surged in recent years, with successful examples such as chlorambucil and bufalin, originally developed as alkylating agents based on the chemical warfare agent mustard gas, later proven effective for treating leukemias [113]. Additionally, thalidomide, despite its notorious history of severe teratogenic effects, has been repurposed for conditions such as leprosy and multiple myeloma. Table 2.1 Drugs proposed for chemoprevention and treatment of skin cancer

Furthermore, arsenic trioxide (a toxic compound) and all-trans retinoic acid (a vitamin A metabolite) were approved by the FDA in 2000 for the treatment of acute promyelocytic leukemia. Thus, drug repurposing emerges as a promising and feasible strategy to expand treatment options in cancer therapy [28].

While several drugs have been identified for potential repurposing in the treatment of skin cancer, the majority have only undergone preclinical evaluation [114, 115]. Comprehensive clinical trials are necessary to establish their efficacy and safety before regulatory approval for this indication. Nevertheless, these agents present a promising alternative, as most are cost-effective and exhibit minimal adverse effects at therapeutic dosages.

2.2.2 Flavonoids

Flavonoids represent a category of polyphenolic secondary metabolites predominantly located in vegetables, tea, soybeans, fruits, and grains [116]. These compounds typically feature aromatic ring structures that constitute their fundamental framework. The chemical

characteristics of flavonoids are influenced by factors such as the degree of hydroxylation, polymerization, conjugation, and other substituents [117]. While individual flavonoid compounds exhibit variability in the substitution patterns on rings the extent of oxidation and substitution patterns on ring C differentiates A and B, distinct flavonoid classes [117]. The biological properties of flavonoid classes are intrinsically linked to their molecular architecture. The flavan core and the substituents' position, quantity, and nature significantly impact their ability to scavenge free radicals and engage in metal chelation [118].

Evidence-based research indicates that flavonoids possess therapeutic potential for a variety of conditions, including cancer, cardiovascular diseases, and immune system disorders [119, 120]. These compounds have been shown to inhibit cellular proliferation and promote both apoptotic and autophagic cell death (Figure 2.8) [121].

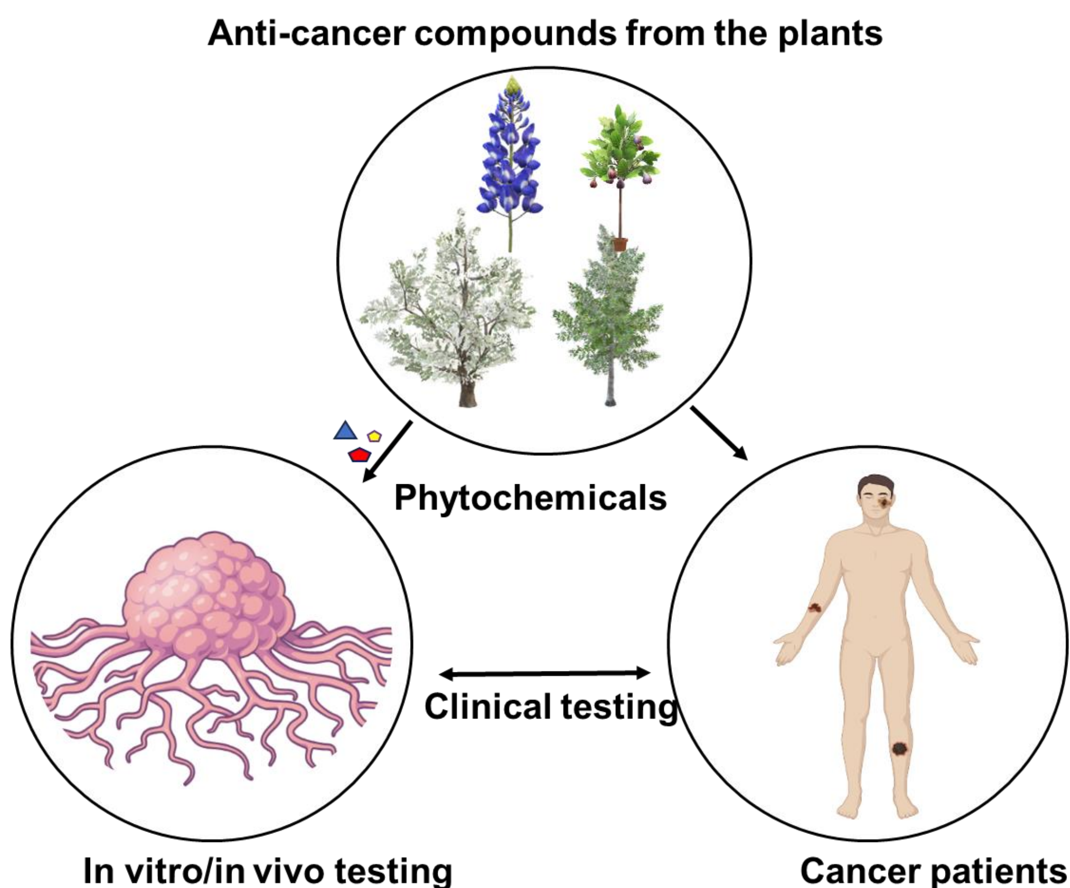

Figure 2.8 Graphical representation of the use of natural products in cancer treatment.

Table 2.2 Summary of flavonoids used in the treatment of melanomas with mechanism of actions

Compound	Cell Line	Effects	Reference
Apigenin	A375P and A375SM	↑ BAX, ↑ p53, ↑ Cleaved caspase-9, ↑ Cleaved PARP, ↓ BCl-2	[122]
	A375 and A2058	↓ p-FAK, ↓ p-ERK-1/2, ↑ Caspase-3, ↑ Cleaved PARP	[123]
	A375 and C8161	↑ G2/M phase (cell cycle), ↑ Cleaved caspase-3	[124]
	A375	↑ Caspase-3, ↑ Caspase-9, ↑ BAX, ↓ BCl-2	[125]
	WM1361B and WM983A	↑ G0/G1 phase (cell cycle), ↓ Cyclin D1/2, ↓ Cyclin E, ↓ CDK2/4/6, ↑ p27Kip1, ↓ pRB, ↓ E2F	[126]
EGCG	B16F10	↓ p-CREB, ↓ CREB, ↓ MITF	[127]
	A375	↑ Caspase-3, ↓ BCl-2, ↑ p-PI3K, ↑ p-AKT, ↑ p-mTOR, ↓ p-AMPK	[128]
Kaempferol	A375SM	↑ p21, ↓ Cyclin E, ↓ Cyclin B, ↑ p38 MAPK, ↑ p53, ↓ BCl-2, ↑ BAX	[129]
	A375	↑ G2/M phase (cell cycle), ↓ mTOR, ↓ PI3K, ↓ Akt	[130]
	B16F10	↑ G2/M phase (cell cycle)	[131]
Naringenin	B16F10 and SK-MEL-28	↓ p-ERK1/2, ↓ p-JNK, ↑ Caspase-3, ↑ Cleaved PARP	[132]
	B16F10	↑ subG0/G1 phase, ↑ S phase, ↑ G2/M phase (cell cycle), ↓ G0/G1 phase	[133]
Silybin	A375 and HS294T	↓ Nuclear β-catenin, ↑ Cytosolic β-catenin, ↓ MMP-2, ↓ MMP-9, ↑ p-β-catenin, ↑ CK1α, ↑ GSK-3β, ↓ BCl-2, ↓ BCl-x, ↑ BAX, ↑ Caspase-9, ↑ Cleaved Caspase-3, ↑ PARP	[134]

Additionally, flavonoids may induce necrosis, arrest the cell cycle, and inhibit processes such as cellular migration, invasion, and tumor angiogenesis, suggesting their capacity to reverse or

mitigate chemoresistance, potentially through the modulation of ROS scavenging enzyme activities [135].

Moreover, flavonoids exhibit significant antioxidant properties, effectively scavenging free radicals and reducing oxidative stress while regulating cellular metabolism. Importantly, they are regarded as environmentally friendly and cost-effective therapeutic agents. A considerable number of flavonoids have demonstrated safety and efficacy in various studies (Table 2.2) [136, 137]. Below, the structures of several flavonoids discussed in this review are presented. The effects of various flavonoids were evaluated in different melanoma cell lines, and a summary of these findings is presented in the table below.

2.3 Dihydroartemisinin

Dihydroartemisinin (DHA) is a semisynthetic derivative produced through the reduction of artemisinin derived from *Artemisia annua* (Figure 2.9) [29]. The C-10 lactone group is replaced with a hemiacetal group in this process. Dimeric forms of DHA have been reported to exhibit activity up to 1,000 times greater than their monomeric counterparts, with their therapeutic mechanism linked to the induction of apoptosis, which subsequently increases the levels of ROS [31].

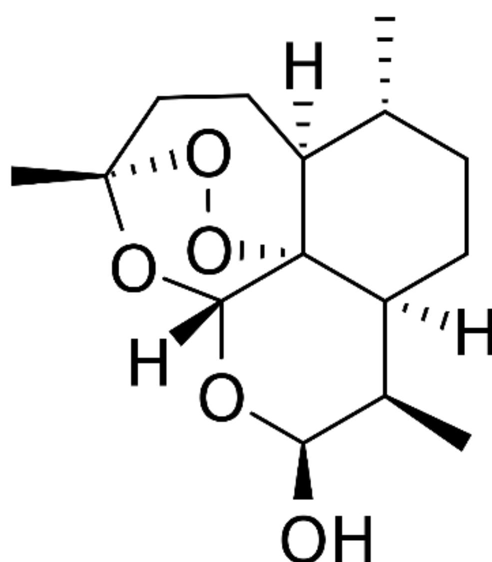

Figure 2.9 Structure of dihydroartemisinin.

DHA is recognized as a first-line antimalarial agent for its enhanced water solubility, efficacy, improved absorption, broader distribution, reduced toxicity, and superior oral bioavailability compared to artemisinin. Recently, there has been growing interest in repurposing DHA due to its potential anticancer properties, although its specific mechanisms remain inadequately characterized [138].

Research indicates that DHA exerts anticancer effects by inhibiting angiogenesis, tumor metastasis, and cellular proliferation. Additionally, it promotes apoptosis, autophagy, and endoplasmic reticulum (ER) stress and modulates immune responses [139].

2.3.1 Physicochemical properties

Table 2.3 Physicochemical properties of DHA

Chemical formula	$C_{15}H_{24}O_5$
IUPAC Name	(3R,5aS,6R,8aS,9R,12S,12aR)-Decahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin-10-ol
Molecular weight	284.3481 g/mol
Solubility	Very slightly soluble in water, soluble in organic solvents such as methanol, ethanol or DMSO
LogP	2.25
pKa	12.11
Oral bioavailability	10-15%

2.3.2 Mechanism of actions in the cancer

DHA exhibits a multifaceted mechanism of action that contributes to its anticancer properties (Figure 2.11).

2.3.2.1 **Induction of Apoptosis**

DHA facilitates apoptosis, or programmed cell death, through the activation of intrinsic apoptotic pathways [140].

Regulation of Apoptotic Proteins: DHA increases the expression of pro-apoptotic proteins such as BAX while simultaneously decreasing anti-apoptotic proteins like Bcl-2. This shift in protein expression leads to mitochondrial membrane permeabilization, resulting in the release of cytochrome c and the activation of downstream caspases (e.g., caspase-3 and caspase-9), ultimately driving the apoptotic cascade [141].

2.3.2.2 **Reactive oxygen species generation**

DHA enhances the production of reactive oxygen species within cancer cells. The elevated ROS levels contribute to oxidative stress, which can cause cellular damage to DNA, proteins, and lipids, leading to cellular dysfunction and death. The oxidative stress induced by DHA is critical in mediating apoptosis in cancer cells [142].

2.3.2.3 **Inhibition of angiogenesis**

DHA exhibits anti-angiogenic properties, disrupting the formation of new blood vessels that supply tumors with nutrients and oxygen via the downregulation of angiogenic factors such as vascular endothelial growth factor (VEGF) and downregulating the matrix metalloprotease (MMP), thereby limiting tumor growth and preventing metastasis [139].

2.3.2.4 **Cell cycle arrest**

DHA can induce cell cycle arrest, particularly at the G1 and G2/M phases, which inhibits cancer cell proliferation. This effect is mediated through the disruption of cell cycle regulators, leading to reduced tumor growth [143].

2.3.2.5 **Immune modulation**

DHA may enhance immune responses, aiding the immune system in recognizing and eliminating cancer cells. This includes the activation of immune cells such as T cells and natural killer (NK) cells, thereby enhancing antitumor immunity [144].

2.3.3 Role of dihydroartemisinin in melanoma

Repurposing DHA, an antimalarial drug derived from artemisinin, has shown promise in cancer treatment due to its ability to induce apoptosis and inhibit tumor growth across various cancer types [29].

Table 2.4 Role of DHA in the treatment of melanoma

Cell lines	**Study (*In vitro*, *In vivo*)**	**Anti-cancer activity**	**References**
B16F10, A375,	*In vitro* and *in vivo*	↓ Lung metastasis ↓ Angiogenesis ↓ Expression of PI3K/AKT/mTOR/HIF-1α/VEGF related pathways	[145]
B16F10	*In vitro* and *in vivo*	↓ Invasion and migration ↑ IFN-γ+CD8+ T $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells and IL-$10^+CD4^+CD25^+$ T cells normalized	[146]
A375, B16F10	*In vitro* and *in vivo*	↓ Invasion and migration ↑ $CD8^+$CTL ↑ Apoptosis ↓STAT3-induced EMT and MMPs	[147]
A375, G361, LOX	*In vitro*	↑ Cellular oxidative stress Activation of cleavage of pro-caspase 3 ↑ oxidative and genotoxic stress response genes	[148]
A 431	*In vitro*	↑ Apoptosis and ROS generation	[149]

This approach leverages the drug's established safety and pharmacological profile, allowing for a faster transition to clinical applications compared to novel drug development [31]. Several

studies have investigated the repurposing of DHA for melanoma treatment [145]. DHA has been shown to inhibit tumor growth and reduce lung metastasis by enhancing the levels of pro-apoptotic factors and modulating pathways associated with PI3K/AKT/mTOR/HIF-1α/VEGF. Additionally, it is effective in decreasing matrix metalloproteinases (MMPs), contributing to its anti-migratory effects [150]. DHA inhibits transcription factors, such as cyclic AMP response element-binding protein (CREB), activated protein-1 (AP-1), nuclear factor-kβ (NF-kβ), and proinflammatory cytokine gene expression (IL-6, IL-1β, GM-CSF, and TNF-α) in B16F10 (melanoma) cells [151].

It also causes G1 phase arrest and apoptosis induction in B16F0 and A375 melanoma cells through activation of checkpoint kinase-1 (Table 2.4) [151]. The DHA was also studied for inhibition of lung metastasis in the B16F10 cell-induced tumor model in C57BL/6 mice [150]. Despite the promising efficacy of DHA in melanoma, its clinical application is limited by factors such as poor aqueous solubility, low oral bioavailability, and hepatotoxicity *in vivo*.

2.4 Hesperidin

Citrus fruits, including oranges, tangerines, lemons, and limes, are widely recognized for their beneficial health effects and potential chemopreventive properties. A notable bioactive compound with promising anticancer activity is hesperidin (HES), also referred to as hesperetin 7-rutinoside (Figure 2.10) [152]. Hesperidin was initially isolated from orange peels in 1828 and was historically misidentified as one of the compounds known as "vitamin P." This polyphenolic glycoside is found in abundance in various citrus fruits [38].

Hesperidin is categorized within the flavanone subclass of flavonoids. Unlike other common flavonoid groups, such as flavones, flavonols, or isoflavones, flavanones lack a double bond between the 2 and 3 positions in the C ring of the typical flavonoid structure [153]. Research has demonstrated that hesperidin possesses a wide array of pharmacological properties, including potent antioxidant, anti-inflammatory, anti-atherosclerotic, cardioprotective,

neuroprotective, anti-allergic, antiviral, antimicrobial, and anticancer effects [38]. Numerous preclinical studies have documented its role in mitigating malignant transformation and tumor progression, highlighting its action through various cellular signaling pathways. Hesperidin can influence multiple molecular targets related to tumor cells' survival, proliferation, and apoptosis.

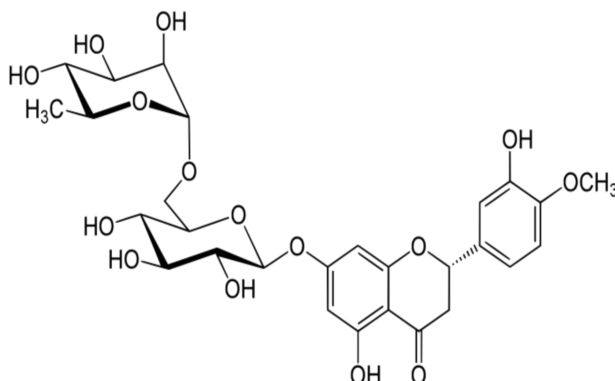

Figure 2.10 Structure of Hesperidin.

2.4.1 Physicochemical properties

The physicochemical properties of HES have been summarized in Table 2.5.

Table 2.5 Physicochemical properties of HES

Chemical formula	$C_{28}H_{34}O_{15}$
IUPAC Name	(2*S*)-3′,5-Dihydroxy-4′-methoxy-7-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyloxy] flavan-4-one
Molecular weight	610.5606 g/mol
Solubility	Very slightly soluble in water, soluble in organic solvents such as methanol, ethanol, or DMSO
LogP	-0.27
pKa	8.61
Oral bioavailability	3-8%

2.4.2 Mechanism of actions

Hesperidin, a flavonoid primarily found in citrus fruits, exhibits several mechanisms that contribute to its anticancer properties (Figure 2.11).

2.4.2.1 **Antioxidant activity**

Hesperidin exhibits strong antioxidant properties that help mitigate cell oxidative stress. This is significant because oxidative stress can lead to DNA damage and promote cancer progression [39].

2.4.2.2 **Inhibition of cell proliferation**

Hesperidin has been shown to inhibit the proliferation of cancer cells by disrupting cell cycle progression. It can induce cell cycle arrest at various phases (such as G1 or G2/M), thereby preventing tumor growth [39, 154].

2.4.2.3 **Induction of apoptosis**

Hesperidin can activate apoptotic pathways in cancer cells. It upregulates pro-apoptotic proteins (Bax) and downregulates anti-apoptotic proteins (like Bcl-2), leading to programmed cell death [155].

2.4.2.4 **Anti-inflammatory effects**

Chronic inflammation is a known contributor to cancer development. Hesperidin exerts anti-inflammatory effects by inhibiting the production of pro-inflammatory cytokines and enzymes (COX-2), which may help reduce tumorigenesis [40].

2.4.2.5 **Inhibition of angiogenesis**

Hesperidin can suppress the formation of new blood vessels (angiogenesis) that tumors need to grow. It inhibits factors such as VEGF, thus starving tumors of nutrients [42].

2.4.2.6 **Modulation of signaling pathways**

Hesperidin interacts with various cellular signaling pathways, including the NF-kB, MAPK, and PI3K/Akt pathways, which are often dysregulated in cancer. By modulating these pathways, hesperidin can influence cancer cell survival, proliferation, and metastasis [42, 156].

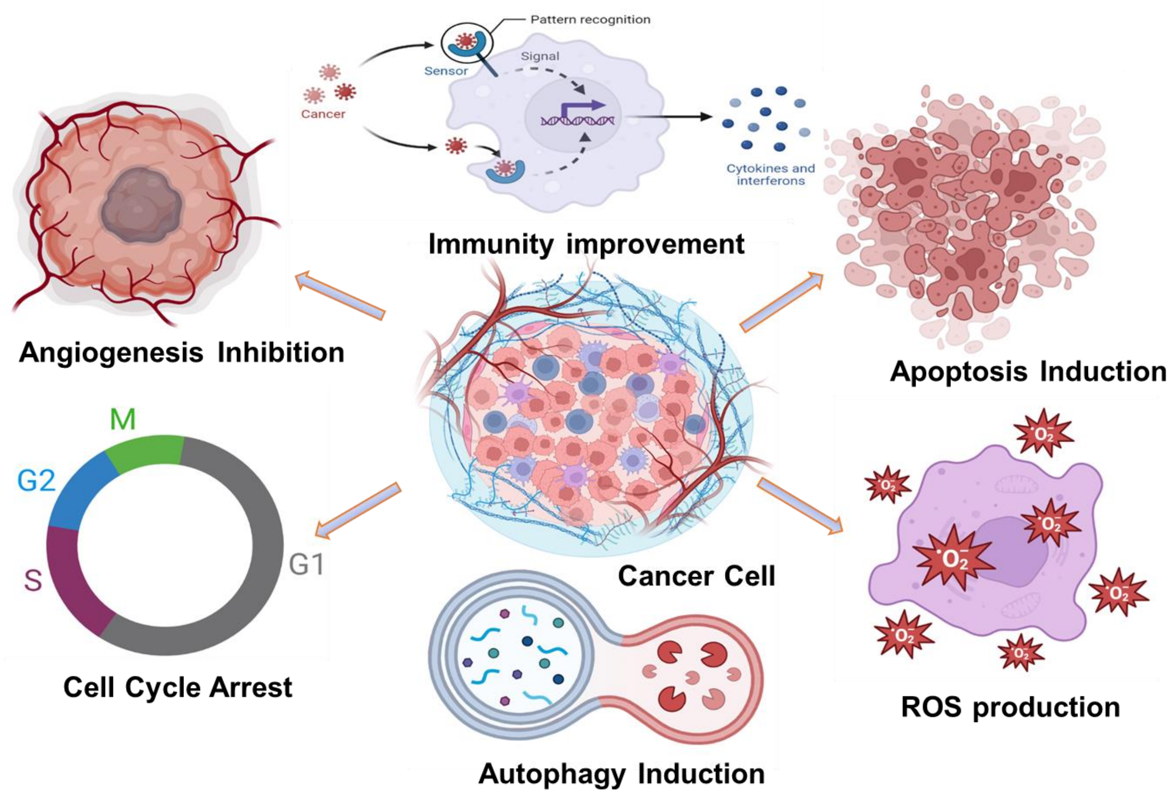

Figure 2.11 Graphical representations of the mechanism of actions of DHA and HES in cancer.

2.4.3 Role of hesperidin in melanoma

Epidemiological studies indicate that high consumption of citrus fruits, which are rich in hesperidin, may be associated with a lower risk of various cancers, including skin cancer, colorectal, and breast cancer [157, 158]. Hesperidin has been explored as an adjunct to conventional cancer therapies. It may help alleviate some side effects of chemotherapy and radiotherapy, such as inflammation and oxidative stress [155]. Moreover, various studies have highlighted hesperidin's effects on different cancer types, showing promise in inhibiting tumor growth and enhancing treatment efficacy, although much of this research is still in preliminary stages [159]. On the other hand, given its natural origin, hesperidin is being investigated as a dietary supplement for cancer patients (Table 2.6). Its inclusion in dietary strategies aims to enhance overall health and support conventional treatment modalities [160]. Nevertheless, the investigation of hesperidin in the context of melanoma has been limited to a small number of studies. To the best of our knowledge and based on a comprehensive literature review, this

represents the first investigation in which hesperidin has been encapsulated within exosomes for the treatment of melanoma.

Table 2.6 Studies on hesperidin and its formulation in melanoma therapy

Cell line	**Study (*in vitro*/*in vivo*)**	**Anti-cancer activity (MoA)**	**Reference**
HT-144	*In vitro*	↑ Increased cytotoxicity ↑ Increased apoptosis ↑ Caspase-3 and Caspase-9	[161]
B16F10	*In vitro* and *in vivo* in C57BL/6	↑ Increased apoptosis ↓ Mitochondrial membrane potential ↑ Generation of ROS ↓ Migration, invasion, and colony formation assay ↓ Expression of metastatic tumor nodules	[159, 162]
A375, CHL01, SKMEL147	*In vitro*	↑ Anti-oxidant activity ↓ Toxicity	139[163]

2.5 Exosomes as a drug delivery vehicle

Exosomes, nanosized extracellular vesicles released by various cell types, have attracted attention as novel drug delivery systems in cancer treatment because of their natural biocompatibility and ability to deliver therapies to specific targets [54-57]. These vesicles can encapsulate a variety of therapeutic agents, such as small molecules, proteins, and nucleic

acids, providing protection against degradation and enhancing their bioavailability (Figure 2.12) [57]. Exosomes possess the ability to preferentially migrate to tumor sites, resulting in increased accumulation within cancerous tissues, which minimizes off-target effects and enhances therapeutic effectiveness [164]. Additionally, their capacity to traverse biological barriers, including the blood-brain barrier, renders them particularly advantageous for targeting difficult-to-treat tumors. Utilizing exosomes for drug delivery presents substantial promise for improving cancer treatment outcomes by facilitating precise and effective targeting of therapeutic agents [165].

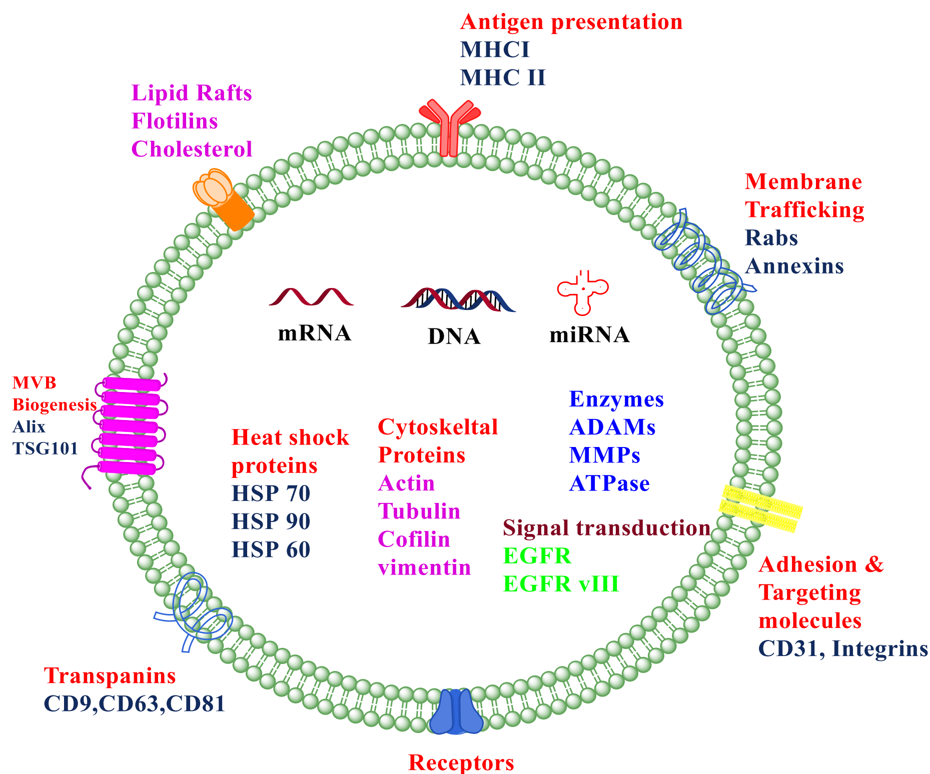

Figure 2.12 Graphical structure of exosomes.

2.5.1 Advantages of exosomes as a drug delivery vehicle

Exosomes offer several advantages as drug delivery vehicles in cancer therapy (Figure 2.13).

Biocompatibility: Being naturally derived from cells, exosomes exhibit low immunogenicity, reducing the risk of adverse reactions when administered to patients [58, 59].

Targeted delivery: Exosomes can preferentially accumulate in tumor tissues due to their inherent ability to recognize and bind to specific receptors on cancer cells, enhancing the precision of drug delivery [59].

Encapsulation of various therapeutics: They can carry a wide range of therapeutic agents, including small molecules, proteins, RNA, and even DNA, facilitating the delivery of diverse treatment modalities [166].

Protection from degradation: Exosomes provide a protective environment for encapsulated drugs, shielding them from enzymatic degradation and enhancing their stability and bioavailability [167].

Ability to cross biological barriers: Exosomes can traverse biological barriers, such as the blood-brain barrier, making them particularly useful for targeting tumors in challenging locations [56].

Biological information transfer: In addition to drug delivery, exosomes can transfer bioactive molecules that modulate the tumor microenvironment, potentially enhancing therapeutic efficacy [165].

Scalability and production: Exosomes can be relatively easily isolated and produced from various cell types, allowing for scalable manufacturing processes [168].

Minimal off-target effects: Their targeted delivery mechanisms help to minimize the impact on healthy tissues, potentially reducing side effects associated with conventional chemotherapy [58].

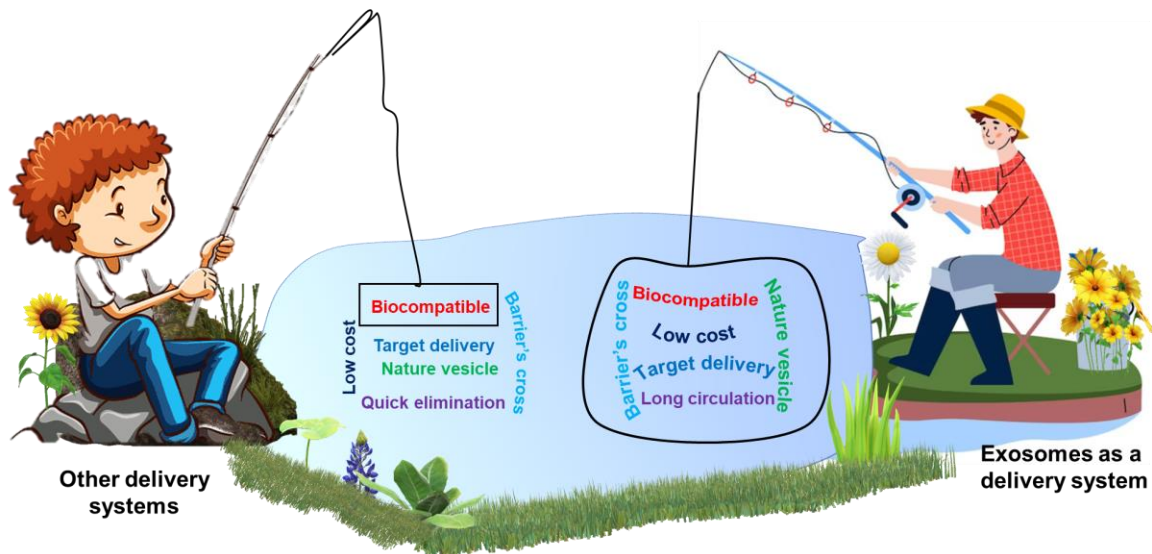

Figure 2.13 Graphical representation of advantages of exosomes compared to other drug delivery systems.

2.5.2 Biogenesis of exosomes

The biogenesis of exosomes may be divided into three major stages: (i) formation of the endocytic vesicle, (ii) formation of multivesicular bodies (MVBs) which comprise intraluminal vesicles (ILVs), and (iii) the fusion of these MVBs with the plasma membrane (Figure 2.14) [60]. The exosomes can be secreted both in a healthy state and a diseased state by a wide array of cell types [56]. During the process of biogenesis, the endocytic vesicle formed from the plasma membrane is first developed into an early endosome and then into a late endosome [55]. The limiting membranes of the late endosomes undergo invagination to produce ILVs within the lumen of organelles. The assembling of ILVs into late endosomes leads to the formation of MVBs. The formation of endocyclic vesicles is controlled by specific proteins that belong to the Rab family [57]. The formation of endosomal vesicles occurs via two pathways namely, endosomal sorting complexes required for the transport (ESCRT) dependent pathway and ESCRT-independent pathway [165]. The Rab family proteins include four ESCRT proteins namely ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III. Apart from the Rab proteins, the other two proteins associated with the transition of endosomes to exosomes include TSG101 and

ALIX. Such a process needs ubiquitination of the cytosolic tail of the endocytosed receptors [56].

TSG101 belongs to the ESCRT-I protein and frames a complex with the ubiquitinated proteins that activate the ESCRT-II protein and promote the oligomerization and formation of the ESCRT-III complex [58]. This complex then gets involved in the sequestration of MVB proteins and the enrollment of the de-ubiquitination catalyst to eliminate the ubiquitin from the load proteins before arranging them into ILVs. In the last stage of exosome formation, the ESCRT-III complex is degraded by vacuolar protein sorting associate protein 4 (VPS4) and ATPase [166]. In ESCRT-independent biogenesis of exosomes, ceramide plays an important role which is synthesized by sphingomylinase enzyme. Inhibition of this sphingomyelinase enzyme reduces the secretion of exosomes in Oli-neu cells [55].

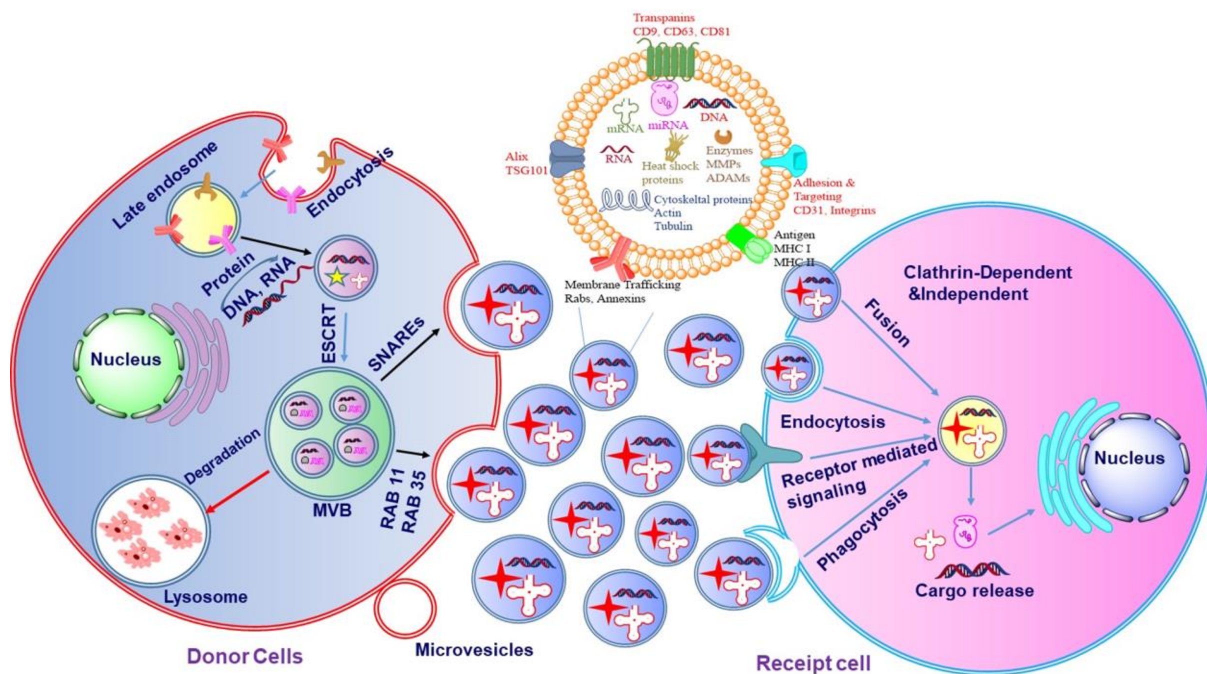

Figure 2.14 Graphical representation of biogenesis of exosomes.

2.5.3 Isolation of exosomes

Several approaches have been reported in the literature for the isolation of EVs, *viz.,* (1) ultra-centrifugation, (2) size-exclusion chromatography, (3) precipitation, (4) ultra-filtration, and (5)

immunoaffinity. Every method has its benefits and drawbacks; however, based on the exosome source and purpose, the method can be adopted for the isolation of exosomes [60].

2.5.3.1 Centrifugation

Two types of centrifugation methods are used to isolate exosomes: (1) differential ultra-centrifugation, separation based on the size of the different components, and (2) density gradient ultra-centrifugation based on their densities.

2.5.3.2 Differential ultracentrifugation

In differential ultracentrifugation, exosomes are isolated by serial centrifugation at different times and speeds. Johnstone et al., 1992 first used the differential ultracentrifugation method to separate exosomes from the tissue culture medium, which was further upgraded by Thery et al., 2006 [169]. As per this report, cell culture supernatant underwent serial centrifugations at 300× *g* and 2000× *g* for each cycle of 10 min, and 10,000× *g* for 30 min to remove the live cells, dead cells, and cell debris, respectively. The final supernatant obtained was ultra-centrifuged at 100,000× *g* for 70 min to obtain the pellets containing exosomes and proteins which were washed with PBS to procure pure exosomes [169].

Although, various research groups have adopted ultracentrifugation-based exosome isolation, however, isolation by using the differential centrifugation method does not yield a large number of pure exosomes which limits its utilization. Therefore, it is advised to either optimize the existing technique or we need to find an alternative method of isolation that will overcome the problems associated with the differential ultracentrifugation method. This may include the optimization of different process variables (number of steps with variable speeds) for obtaining the pure population of exosomes.

2.5.3.3 Density gradient ultracentrifugation

In the density gradient ultracentrifugation method, similar steps are being followed as the differential ultracentrifugation method except for the last stage where the

separation/purification is completed in high-density sugar solution or iodixanol which leads to the collection of pure exosomes at the middle layers of the test tube.

2.5.3.4 **Chromatography-based isolation method**

In this method, exosomes are separated based on size differences between the EVs in biological samples by using size-exclusion chromatography (SEC), which is also known as gel filtration or molecular sieve chromatography. The source material of the EVs is loaded on the column packed with Sepharose©, Sephacryl©, or BioGel P, as a stationary phase [165]. Thus, the elution is based on their size in the order of decreasing molecular weight. The commercially available columns that are employed to isolate the exosomes include qEV separation columns, EV second purification columns, Exo Cap™, and Exo-spin. Compared to ultracentrifugation and ultrafiltration, the chromatography-based isolation method is quick and of relatively low cost. Additionally, the isolated fractions are uniform in size, and their biological characteristics remain significantly unaffected by this method [60]. However, there are some chances where exosomal fractions may get contaminated with other particles of the same size which may compromise the purity of exosomes. Nevertheless, owing to its time-consuming process, the SEC method limits its use in treatment and research.

2.5.3.5 **Ultrafiltration-based isolation**

Ultrafiltration-based isolation of exosomes depends upon the separation of the different classes of EVs, such as apoptotic bodies, microvesicles, exosomes, and other contamination proteins based on their different sizes [170]. The filtration-based isolation method is classified into three types: ultrafiltration, hydrostatic dialysis, and gel filtration. In the ultrafiltration method, the ultrafiltration membranes are used to isolate exosomes that are available in different pore sizes, such as 0.8, 0.45, 0.22, and 0.1 μm capable of retaining the particles with diameters of 800, 450, 220, and 100 nm, respectively. Larger particles, such as apoptotic bodies, are separated first, followed by microvesicles and exosomes [60].

2.5.3.6 **Precipitation-based isolation**

The precipitation-based isolation method is widely used for the isolation of exosomes. As per an earlier report, precipitation-based strategies are utilized particularly for biologics having a low initial volume [171]. It was observed that 84% of scientists utilize the precipitation method for investigating the encapsulation of RNA in EVs. Polymers such as polyethylene glycol (PEG), salt solutions such as sodium acetate, organic salts, and a charge-based moiety such as protamine are used in the precipitation method [60].

2.5.3.7 **Immunoaffinity-based isolation**

Immunoaffinity is a separation and purification method based on certain interactions between antibodies and antigens to isolate the required component from a mixture. This is the most promising method for the isolation of specific exosomes. The commonly used antibodies in immunoaffinity-based isolation are monoclonal antibodies against specific biomarkers: CD9, CD63, CD80, and TSG101 for exosomes, integrins and CD40 for microvesicles, and annexin V for apoptotic bodies [60]. These antibodies are fixed on different types of materials such as magnetic beads or microfluidic devices. Nakai et al., 2016 isolated exosomes from mouse peritoneal macrophages cells. For this, Tim4-FC protein was used to bind with phosphatidylserine present on the surface of the exosomes which were then captured by using magnetic beads [172]. However, immunoaffinity-based isolation is not suitable for isolating a large number of exosomes. Moreover, the high cost and low yield of exosomes limit their usefulness.

2.5.4 Drug loading methods

Exosomes can be used as nanocargos that are capable of loading different small and large molecules. The drug can be loaded either before or after the isolation of exosomes, depending on the application. Each of these loading methods has its pros and cons (Figure 2.15 and Table 2.7).

Drug loading before the isolation of exosomes can be performed in two ways: (1) incubation of parent cells with a drug and (2) by gene editing. In the incubation method of drug loading, the compounds are directly mixed with the cell culture medium, where the drugs get internalized into cells and are loaded into EVs via an endogenous mechanism [60].

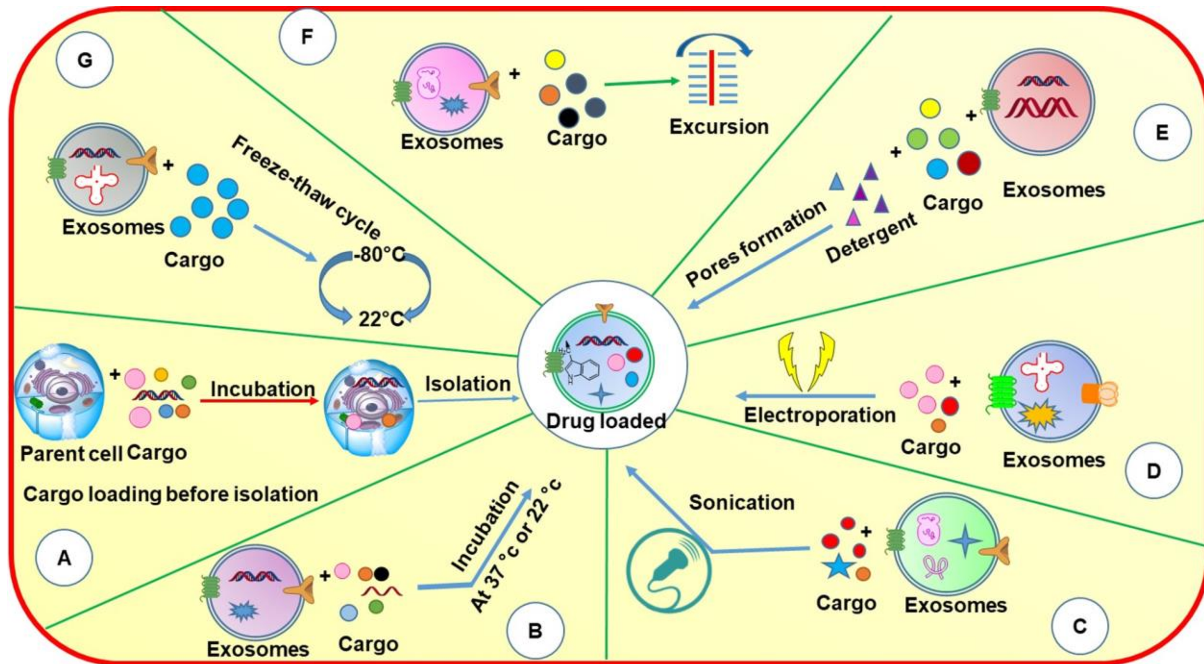

Figure 2.15 Schematic diagram showing different methods of loading cargos into exosomes through pre-loading and post-loading strategies: (**A**) incubation with parent cell; (**B**) incubation; (**C**) sonication; (**D**) electroporation; (**E**) detergent method; (**F**) excursion; (**G**) freeze-thaw cycle.

2.5.4.1 **Drug loading after isolation of exosomes**

Several methods have been employed for drug loading after exosome isolation. These include incubation, sonication, detergent treatment, electroporation, extrusion, and freeze-thaw cycles, all of which are used to load drugs into exosomes following their isolation from the respective biological source. These loading techniques have been adapted from the field of liposomes, as exosomes are considered analogous to liposomes but offer higher stability and enhanced biocompatibility. Except for incubation, the other methods rely on the assumption that the exosome's lipid bilayer will reassemble in its original form, similar to liposomes, after the application and subsequent removal of stress. While this assumption may hold true, the impact

on the biological activity of integral proteins and their ability to realign in their original positions still requires further investigation. Each method has its own strengths and limitations, but incubation is often considered the simplest and safest drug-loading technique, as it is less likely to alter the exosome's structure or its various components [60]. A summary of the different loading methods is provided below.

Table 2.7 Summary of different drug loading methods into exsomes

Method	Mechanism	Advantages	Disadvantages
Incubation before isolation	Drugs are internalized into cells and loaded into the vesicles by the endogenous mechanism of cells	Useful for hydrophobic drugs, does not affect the integrity of exosomes	Not useful for hydrophilic drugs, low drug loading capacity
Incubation after isolation	Passive diffusion method	Simple method, useful for hydrophobic drugs, does not affect the integrity of exosomes	Not useful for hydrophilic drugs, low drug loading capacity, cannot load nucleotides
Sonication	The mechanical shear force from the sonicator probe decreases the membrane integrity and forms the pores	A large amount of drugs can be loaded	Can damage the proteins, other components, and integrity
Electroporation	Applying a high-voltage electrical charge to create temporary pores on the exosomal membrane	Useful for loading of hydrophilic drugs, siRNA, and miRNA	Possible siRNA precipitation and aggregation or fusion of vesicles.
Detergent treatment	Selectively forms a complex with cholesterol bound to exosomal membranes to form a porous structure on the membrane surface	More loading capacity compared to electroporation	Detergents such as Saponin may show hemolysis *in vivo*
Freeze-thaw cycles	Repeated cycles of freezing and thawing may cause the stress-induced formation and deformation of exosomes vesicles leading to encapsulation of the drug during this process	Industrial acceptable technique for the preparation of the liposomes and the same can be adopted for exosomes	The drug loading capacity is usually lower than that of sonication and extrusion
Extrusion	Mechanical stress during the extrusion process may disrupt the membrane which may be resealed following the extrusion by allowing the drug to get entrapped during this process	Industrial acceptable technique for getting the liposomes with uniform distribution	However, it may damage the membrane structure of exosomes which may cause drug leakage