Biochimica et Biophysica Acta 1846 (2014) 312–325

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbacan

Review

Deconstructing breast cancer cell biology and the mechanisms of multidrug resistance

Mafalda Videira [a,b,*], Rita Leones Reis [a], Maria Alexandra Brito [a,c]

[a] Research Institute for Medicines (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, 1649-003 Lisbon, Portugal
[b] Department of Galenic Pharmacy and Pharmaceutical Technology, Faculdade de Farmácia, Universidade de Lisboa, 1649-003 Lisbon, Portugal
[c] Department of Biochemistry and Human Biology, Faculdade de Farmácia, Universidade de Lisboa, 1649-003 Lisbon, Portugal

ARTICLE INFO

Article history:
Received 19 June 2014
Received in revised form 21 July 2014
Accepted 22 July 2014
Available online 28 July 2014

Keywords:
Cancer biology
Chemoresistance
Efflux transporters
Epithelial-mesenchymal transition
Multidrug resistance proteins
Tumor microenvironment

ABSTRACT

Cancer complexity constantly challenges the way that clinicians manage breast cancer therapy. Tumor heterogeneity and intratumoral stroma characteristics allow cells with different phenotypes and deregulated apoptotic, proliferative and migration abilities to co-exist contributing to a disappointing therapeutic response. While new approaches are being associated with conventional chemotherapy, such as hormonal therapy or target monoclonal antibodies, recurrence and metastasization are still observed. Membrane transporters are the cell's first line of contact with anticancer drugs having a major role in multidrug resistance events. This structural-based activity enables the cell to be drug-resistant by decreasing drug intracellular concentration through an efflux-transport mechanism, mainly associated with overexpression of ATP-binding cassette (ABC) proteins. This review focuses on some of the important structural and biological properties of the malignant cell and tumor microenvironment, addressing the role of the membrane ABC transporters in therapeutic outcomes, and highlighting related molecular pathways that may represent meaningful target therapies.

Contents

Abbreviations: ABC, ATP-binding cassette; BC, breast cancer; BCRP, breast cancer resistance protein; CAFs, cancer-associated fibroblasts; CSC, cancer stem cells; ECM, extracellular matrix; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; HIF-1α, hypoxia inducible factor-1α; MDR, multidrug resistance; HGF, hepatocyte growth factor; mTOR, mammalian target of rapamycin; LOX, lysyl oxidase; MAPK, mitogen-activated protein kinase; MMPs, matrix metalloproteinases; MRP, multidrug resistance-associated protein; NBD, nucleotide binding domain; NF-kB, nuclear factor kappa B; NOS, nitric oxide synthase; P-gp, P-glycoprotein; PI3K, phosphoinositide 3-kinase; PR, progesterone receptor; TAMs, tumor associated macrophages; TGF-β, transforming growth factor-β; TMDs, transmembrane domains; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor

* Corresponding author at: Faculdade de Farmácia, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal. Tel.: +351 919539400; fax: +351 217946491.
E-mail address: mvideira@ff.ul.pt (M. Videira).

http://dx.doi.org/10.1016/j.bbcan.2014.07.011
0304-419X/

1. Introduction

While some researchers are looking at the future of breast cancer (BC) as a chronic disease, some scientists are skeptic regarding this optimistic point of view. In fact, Europe cancer epidemiology ranks breast carcinoma as the second major cause of death, with an incidence of 464,000 new cases in 2012 [1]. Chemotherapy, surgery, radiotherapy, hormonal therapy and targeted antibodies have contributed to a huge increase in patients' survival rate in the last decade. This general trend is very impressive, but in more aggressive and advanced stages these strategies are characterized by poor outcomes, lack of specificity and poor avoidance of recurrence [2].

A general stratification in four stages based on the age, node involvement, tumor grade and size is generally applied to design a therapeutic approach. Stage I is a well-defined and localized tumor mass, characterized by poor invasion properties. Stages II and III correspond to an increase in the tumor volume and to the acquisition of an invasive phenotype. Lymph node involvement gives rise to a sub-classification in A, B and C. Advanced tumors are classified in stage IV. Huge tumor size and metastasis dissemination in main organs, such as lungs and bone, are correlated with this invasive phenotype. This dissemination pattern has critical implications in tumor eradication therapeutic success [3,4]. Disease progression occurs together with several acquired mutations involved in chemoresistance and activation of tumor growth and survival providing heterogeneous clinical outcomes. In this challenging scenario, great efforts have been devoted to the study of the cell molecular mechanisms underlying tumor heterogeneity and multidrug resistance (MDR) aiming at more effective, rational and disease-orientated strategies.

A major breakthrough was the disease classification based in the expression of human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR) and of estrogen receptor (ER). Four different disease subtypes were classified according to these cell marker expressions: ER +/HER2 −, ER +/HER2 +, ER −/HER2 +, and triple negative, which has none of the above receptors ER −/PR −/HER2 − [5]. Although a new genome-driven classification has been pursued [6], the heterogeneity raised by the current accepted classification may explain differences in tumor proliferative capability or the resistance to therapy.

Despite the tumor stage and combinatory therapy approach, patient survival strongly depends on prior chemotherapy and this is tempered by the early adaptive drug escape. It is now clear that the overexpression of ATP-binding cassette (ABC) efflux transporters in malignant cancer cells pump out drug molecules, decreasing their intracellular concentration and the drug pharmacological effect, while increasing the healthy cells' drug exposure [7,8]. A literature overview indicates that this mechanism does regulate cancer survival and progression, directly or indirectly through a non-genomic mediated pathway. Probably, other molecular pathways implicated in drug resistance, such as inhibition of DNA repair and deregulation of survival/apoptotic pathway are tightly related to the MDR signaling activation [9,10]. Thus, better options for cancer treatment can be driven by newly gained knowledge in cancer biology and the main mechanisms involved in tumor progression and resistance, including the role of the most relevant ABC members: P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP) and breast cancer resistance protein (BCRP).

2. Understanding breast cancer biology

At normal physiological conditions, the epithelium is a well-defined structure, where epithelial cells form a uniform layer. In the particular case of the breast, epithelial cells form the lining of ducts that are responsible for milk transport during lactation. Ductal carcinoma is the most common type of BC, occurring both in women and men, although its prevalence in men is rare [11].

Contrasting with healthy tissues, cells within solid tumors acquire several pathophysiological characteristics (Fig. 1) that confer them survival, proliferative and migratory capabilities. Tumor progression is characterized by a mass formed by multiple populations of cells

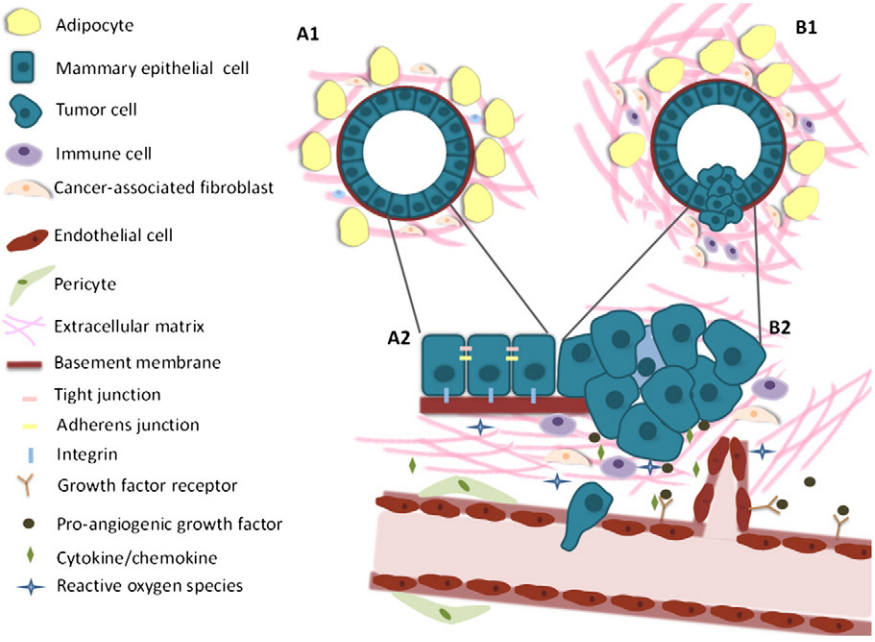

Fig. 1. Schematic representation of key alterations occurring in breast cancer. Polarized mammary epithelial cells lining a duct in a compliant extracellular matrix (ECM) in normal breast (A1); establishment of normal intercellular junctions (tight and adherents junctions) between neighboring cells and attachment to basement membrane by integrins promote contact inhibition, constrain epithelial cell proliferation and assure polarity in normal breast (A2). A duct in breast cancer where mammary epithelial cells proliferate, migrate and lose the polarized structure promoted by a collagen dense matrix in breast cancer (B1); the tumor microenvironment is a complex scaffold of ECM, cancer cells and various other cell types, such as cancer-associated fibroblasts and immune cells, that promotes self-sufficiency in growth signals, as well as a pro-inflammatory and pro-oxidant environment; crucial for tumor development is the increased angiogenesis that is characterized by hyperpermeable blood vessels that are poorly ensheathed by a basement membrane and pericytes; as the distance from blood vessels increase, the environment becomes increasingly hypoxic (lighter cell) (B2).

with mechanisms capable of inhibiting apoptosis, while promoting survival pathways and the invasion of healthy tissues through the blood and lymphatic circulation. Actually, morphologic and functional alterations of epithelial cells, as well as alterations of important surface molecule expression, are crucial for tumor initiation and development. Additionally, tumor microenvironment properties and events such as intratumoral hypoxia, angiogenesis, the epithelial–mesenchymal transition (EMT) program and tumor immunosuppressive properties, are pivotal events in cell malignancy. Generally, a more invasive phenotype is associated with multiple molecular signaling molecules, such as phosphoinositide 3-kinase (PI3K), vascular endothelial growth factor (VEGF), hypoxia inducible factor-1α (HIF-1α) and the EMT related proteins, as well as with the way they all communicate. Taking this in consideration, the tumor microenvironment and related metabolism, the role of hypoxia, EMT and cancer cell heterogeneity in tumor development and progression, as well as the pathways behind such events will be discussed below, addressing their relevance to cancer therapeutic failure and cancer recurrence. Particular emphasis will be devoted to the mutation in the PI3K/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) axis that is frequently deregulated in BC and is significantly correlated with resistance to therapeutic agents. Understanding this signaling pathway frequently deregulated in cancer and its clinical implications could be used to selectively address cell malignancy. Furthermore, multidisciplinary scientific knowledge for advanced therapies especially those related to carrier-mediated delivery innovative strategies could also be leveraged. This topic is comprehensively reviewed elsewhere [12].

2.1. Tumor microenvironment: implications in cell malignant phenotype

The tumor microenvironment is a complex dynamic network, composed of cancer cells, stromal cells and extracellular matrix (ECM), as schematically depicted in Fig. 1, with an increasing importance in solid tumors. Cancer cells are characterized by an adaptive phenotype, responsible for their survival under severe conditions, such as low levels of oxygen and nutrients. Part of this adaptive phenotype is justified by genetic instability and high levels of mutations of cancer cells, important factors that promote malignancy through deregulated survival and proliferation pathways. Besides the altered genetic patterns of cancer cells, malignancy and invasive properties also depend on the surrounding environment, which is involved in tumor initiation, progression and metastasis [13].

Stromal tissue is a supportive tissue composed of several types of cells, such as fibroblasts, vascular (e.g. pericytes) and immune (e.g. macrophages) cells, capable of dynamic crosstalk with cancer cells [14]. Fibroblasts recruited to the tumor area, denominated as cancer-associated fibroblasts (CAFs), are activated and synthesize a large range of factors that acts in an autocrine and paracrine way. Transforming growth factor-β (TGF-β) is one of the factors released by CAFs, being related to the acquisition of EMT when the activation of signaling pathways such as mitogen-activated protein kinase (MAPK) and Smad occurs. CAFs are also related to epithelial cell migration through hepatocyte growth factor (HGF) release, a chemoattractant that guides cancer cell movement to other organs [14]. Moreover, the involvement of inflammation processes in cancer is related to a more malignant progression. Recruitment of monocytes differentiated as tumor associated macrophages (TAMs) in the tumor tissue promotes tissue remodeling, immune suppression and tumor progression through the secretion of several cytokines, growth factors and proteases such as tumor necrosis factor-α (TNF-α), VEGF, and epidermal growth factor (EGF), as well as of matrix metalloproteinases (MMPs), a family of extracellular matrix-degrading enzymes. Furthermore, nitric oxide synthase (NOS) up-regulation is associated with vasodilatation, enhancing blood flow in the tumor microenvironment [15–17]. As perivascular stromal cells, pericytes are important in structural support, stability and formation of blood vessels. However, the role of pericytes in tumor biology is still unclear due to the little knowledge about their recruitment and interaction with other stromal or tumor cells [18]. Moreover, reliable and specific markers of pericytes are still lacking [19], which further renders difficult the unequivocal identification of these cells.

A major part of the tumor microenvironment is the ECM which is composed of macromolecules with different physical and biochemical properties, such as collagen, elastin, fibronectin and proteoglycans, and is tightly controlled in organ development and function [20]. These components, many of them synthesized by fibroblasts, are part of the constitution of basement membrane and the ECM, providing tissues with mechanical and structural support while ECM biochemical properties retain signaling capabilities that promote microenvironment communication [20,21]. Nevertheless, the ECM becomes deregulated and disorganized in cancer conditions, a process initiated through ECM remodeling by MMPs. MMPs are overexpressed in BC, especially MMP-2 and MMP-9, and are related to EMT through enhanced ECM digestion and decreased cell–cell adhesion via E-cadherin degradation, promoting cancer cell transition through physical barriers and healthy tissue entrance [22,23]. Additionally, growth factor cleavage from the ECM and modulation of signaling pathways such as TGF-β and VEGF amplify growth and migration processes of tumor cells and correlate MMPs with inflammatory, angiogenesis, cell proliferation and migration processes [23].

Reorganization of the ECM in tumors is related to collagen, a fibrous protein with an enhanced deposition and deregulated organization in BC. In fact, collagen organization in parallel and perpendicular fibers promotes invasiveness of cancer cells and the activation of collagen receptors, such as discoidin domain receptor, is related to Snail stabilization and BC metastasis [24,25]. The increased ECM remodeling and metastasization in cancer is mediated by lysyl oxidase (LOX), an enzyme implicated in the cross-linking of collagens and elastin [26,27]. Moreover, the increased collagen deposition upregulates integrin signaling, which in turn induces cell proliferation and survival. Integrins are transmembrane receptors for ECM proteins such as collagen and fibronectin, capable of information transition between cancer cells and the ECM, maintenance of cell polarity and modulation of important pathways, such as nuclear factor kappa B (NF-kB), that lead to altered cancer cell apoptosis [28]. As regulators of tyrosine kinase pathways, such as the PI3K pathway that will be discussed below, integrins are involved in initiation, progression and metastasis of solid tumors. Furthermore, their expression in vascular endothelium, immune cells, monocytes and fibroblasts, besides cancer cells, promotes angiogenesis and growth factor secretion, with further consequences in deregulation of proliferative and migration processes [29,30]. With an increased interest in ECM composition, function and involvement in cancer, a large range of new therapeutic targets is being identified, such as integrins and LOX, leading to new perspectives in cancer therapy. The complexity of the tumor microenvironment, characterized by the components above described is transverse to all solid tumors, such as BC.

2.1.1. The role of hypoxia in tumor metabolism

Cellular metabolism within solid tumors has considerable differences from healthy tissues, supporting cancer cell viability through alternative energetic pathway activation. The altered metabolism in cancer cells is characterized by oxidative phosphorylation suppression and glycolysis activation, as well as glutamine use. Decreased mitochondrial function and enhanced glycolysis followed by lactic acid fermentation, a low efficient ATP production process, increases cancer cell dependence on glucose and provides a metabolic process capable of energy production in hypoxia conditions [31,32]. The switch from aerobic to anaerobic metabolism is denominated as the Warburg effect [33], which is observed even in oxygen presence and is characterized by a dynamic hypoxia condition, resulting in periods of hypoxia and reoxygenation.

The impairment of normal blood flow due to hypoxia triggers the activation of transcription factors and signaling pathways related to angiogenesis and cell survival that promote cancer cell adaptation to a more hostile environment. Intratumoral hypoxia stabilizes HIF-1α, a master regulator of O_2 homeostasis [34] and a transcription factor responsible for overexpression of glucose transporters and glycolytic enzymes, as well as blocking mitochondrial oxidative phosphorylation [31,35]. HIF-1α also activates the transcription of genes responsible for the shift from aerobic to anaerobic metabolism, essential to tumor proliferation in hypoxic conditions [14]. Among the more than 70 genes activated by HIF-1α, are the ones related to glucose transporter 1, HER2, ER, VEGF, NOS, EGF and drug efflux transporters [14,36]. Some of these signaling cascades, such as overexpression of HER2 and loss of ER, are directly implicated in the development and severity of BC, and are responsible for a more aggressive phenotype and decreased treatment response [37,38]. Furthermore, hypoxia conditions induce alterations in the tumor microenvironment, with major consequences on tumor metastasis, through upregulation of LOX and MMP-9 and cell survival via alterations in DNA structure and function [39,40]. DNA damage and decreased repair of damaged DNA, amplification of mutations and DNA single and double stand breaks also occur as a result of reactive oxygen species formation along successive periods of hypoxia and reoxygenation.

Decreased pH in the tumor microenvironment as a result of anaerobic metabolism and accumulation of lactic acid has treatment implications, since ionization of weakly basic drugs prevents the cross of cell membranes, thus decreasing therapeutic effect [41,42]. Moreover, cancer cell survival requires glutamine, an amino acid that acts as a precursor of macromolecules essential for cell proliferation. Glutamine production is also associated with CAFs, which suggests that the tumor microenvironment supports cancer cells' altered metabolism [43,44].

2.1.2. Angiogenesis, a step towards invasive disease

As the tumor grows, a vascular network supporting the cancer environment increases due to the development of new vessels in response to VEGF production, a process named angiogenesis [45]. Indeed, tumor tissues are pro-angiogenic environments, where uncontrolled production of angiogenic promoters and a decreased production of inhibitors of angiogenesis occur (Fig. 1). This process leads to structurally and functionally abnormal vessels, characterized by an altered architecture with irregular shapes and fenestrations, which result in enhanced microvascular permeability [46,47]. Moreover, tumor-associated microvessels are characterized by diminished pericyte vascular coverage and a perturbed association between pericytes and endothelial cells leading to aberrant tumor microvessels [48]. In consequence of the reduced consistency of tumor vessels and the poor vascular architecture, the blood flow near cancer cells is variable, causing compromised oxygen and nutrient delivery [49,50].

Pericyte recruitment along endothelial cells is essential for tumor vessel formation and is related to several signaling pathways, namely platelet-derived growth factor B/platelet-derived growth factor receptor-β, sphingosine 1-phosphate/endothelial differentiation gene-1, angiopoietin 1/Tie2 (a receptor tyrosine kinase) and TGF-β/activin-like kinase receptor [51,52]. However, the involvement of such signaling pathways in tumor angiogenesis remains to be clarified. Pericyte recruitment also depends on MMPs, such as MMP-4 and MMP-9, that degrade ECM and promote the release of growth factors, thus stimulating pericyte proliferation and invasion, which could play a role in tumor vessel maturation [52,53]. Recent evidence points to perivascular invasion, or pericyte mimicry, as a distinctly different mode of tumor spread relying on migration along the outer or abluminal aspect of vessels, which has been observed in several malignancies [54]. Furthermore, increased hypoxia and EMT are processes related to abnormal number of pericytes through signaling pathways such as Met, the receptor for HGF [18,55], and it was proposed that the analysis of pericyte vascular coverage of tumor vessels coupled with Met expression could serve as a useful biomarker of patient prognosis [55]. In addition, vascular mimicry, a process where tumor cells acquire endothelial characteristics, occurs, coating integrally vascular vessels and synthesizing a large number of molecules, such as VEGF. Moreover, VEGF is considered an important target in therapy because of its involvement in processes such as permeability, migration and proliferation [56,57].

2.1.3. Exosomes: nanovesicles that drive invasive phenotype

The tumor microenvironment relies on a dynamic flow of information through which different cells sharing the same compartment communicate. Messages based in macromolecules or oncogenes are sent through mediators known as exosomes, which are nanovesicles (50–100 nm) biosynthesized in intracellular compartments related to late endosomes from viable cells, including tumor cells [58,59]. Currently, much attention is being given to these cell nanotechnology-based phenomena intended to deliver information that modulates important signaling pathways of recipient cells both in the tumor microenvironment and at distant metastasis compartment, promoting tumorigenesis and metastatic spread. Circulating exosomes, loaded with lipids, genetic material (mRNA, miRNA) and proteins, as well as with a number of heparan sulfate proteoglicans (e.g., heparanase) and their binding partners (e.g., FGF, VEGF and HGF), have been implicated in tumor progression, through the dissemination of oncogenes and drug-resistance associated macromolecules [58,60–62]. Up to date knowledge emphasized their composition, namely raft-associated lipids as cholesterol and ceramides, distinct from other cell vesicles and responsible for their ability to merge with recipient cells. Apart from their cargo, their interaction with specific cells may also be related to their unique surface membrane proteins [63]. Additionally, cell-type specific markers generated from a variety of viable cells, such as CD3 on T cell exosomes, have been isolated and identified [64]. Their presence in the tumor microenvironment and further involvement in angiogenesis, chemoresistance, metastasization and especially in setting the scene for an immune-privileged environment are well established. More challenging is gaining information on the underlying mechanisms that control genetic signaling messages and their role at the interface of two crucial cancer cell tools: tumorigenicity and immunosuppressive properties. Recent investigations showed that exosomes secreted by triple negative BC cells are powerful mediators of receptor cell metabolic alterations [65].

Crosstalk was observed upon exosome secretion from fibroblasts showing a cell migration promoting role in BC cells through activation of Wnt-planar cell polarity signaling, a pathway associated with cell metastatic and angiogenic modulation [61,66,67]. Likewise, TGF-β expressed at the surface of BC-derived exosomes has been implicated in stromal fibroblast differentiation with central implications in tumor invasion and progression [68]. Also dendritic cell differentiation blockage was achieved through the increase in IL-6 expression promoted by secreted exosomes [69]. In another example, a pro-inflammatory phenotype induction in mammary gland fibroblasts was classified as a crosstalk driven event triggered by hypoxic MCF7 derived exosomes [70].

Due to the exosomes' biological complexity, more insights on their variety and composition is crucial to understanding their role in cancer thus eliciting new interesting therapeutic strategies. In this regard, attention has been directed to heparanase, an endoglucuronidase that cleaves heparan sulfate chains of proteoglycans, leading to a robust up-regulation of growth factor expression so that therapies targeting heparanase hold promise for blocking the behavior of cancer [62].

2.2. Epithelial-mesenchymal transition

Cell dissemination occurs when epithelial membrane becomes disorganized and polarity, as well as cell–cell contacts are lost, enabling cell detachment and migration (Fig. 1). This EMT program is

characterized by the increase of mesenchymal signaling components such as vimentin and N-cadherin and alteration of several membrane and intracellular proteins, such as the decrease of E-cadherin, a transmembrane glycoprotein responsible for cell–cell adhesion and epithelial/endothelial polarity [71]. This is an important process in embryonic development but their deregulation plays a crucial role in cancer progression being critical in the transition from a non-invasive to an invasive cancer phenotype, including in BC [72].

In physiological conditions, transmembrane E-cadherin and intracellular β-catenin are responsible for the maintenance of epithelial integrity by forming a complex that binds to cytoskeleton actin. In tumor cells, the absence of these proteins together with the expression of mesenchymal markers, such as vimentin and fibronectin, is associated with morphological changes correlated with the cell EMT being therefore considered hallmarks usually correlated with tumor migration, invasiveness and metastasization [73–75]. Several transcriptional factors such as Twist, Snail, Slug and NF-kB are EMT modulators, repressing gene expression of epithelial proteins and promoting mesenchyme-related protein expression [76,77], whereas MAPK, p38, TGF-β and PI3K are some of the signal transducers involved in EMT transcriptional regulation [78–80].

Another interesting factor in the malignant compartment is the existence of a specific sub-population: the cancer stem cells (CSC). This theory defends an existent hierarchy in tumors where a subpopulation of CSC is the center of this organized population and experiences self-renewal by cell division, originating a variety of cells that form the tumor [81–83]. CSC also exhibit a rich cell-phenotype heterogeneity capable of different responses to many stimuli giving rise to acquired chemoresistance and supporting the invasiveness and malignancy of recurrent tumors [84].

Breast CSC, bearing the $CD44^{+}/CD24^{-}$ receptor can undergo EMT, forming multicellular spheroids or mamospheres able to reconstruct the original tumor phenotype by a differentiation process that results in an adherent epithelial lineage with improved migratory and invasive potential [85–87]. Cell membrane decorated with CD44 may serve to cluster cancer sub-populations and more important to serve as therapeutic target.

2.3. Cancer-related signaling pathways implicated in tumorigenesis

Cancer cells dictate the biological mechanisms relevant to cell survival and malignant phenotype acquisition potentiating cell invasive adaptive properties. Simultaneously, cell mediators shape the tumor microenvironment and the ECM in order to influence metastasis dissemination. At this point, tumorigenic properties of invasive BC cells prevail and multiple deregulated pathways and their epigenetic regulation can no longer be restrained. Several significant cellular checkpoints in these tightly regulated mechanisms can be therefore selected as targets for cancer therapeutics. Among them, the PI3K/Akt/mTOR axis that is related to tumor growth, cell survival, EMT program and proliferation [88,89] was found to modulate some of the most relevant signaling pathways implicated in tumorigenesis (Fig. 2).

Membrane growth factor receptors (receptor tyrosine kinases), such as EGF receptor, HER2, insulin growth factor receptor and G-protein-coupled receptors are responsible for the intracellular PI3K activation that catalyzes the passage of phosphatidylinositol diphosphate to phosphatidylinositol 3,4,5 triphosphate and leads to Akt (mainly Akt2) pathway activation. The Akt family has multiple targets that lead to cell proliferation and survival, such as mTOR, which promotes cell cycle progression and inhibits pro-apoptotic members of the BCL-2 family, for example Bcl-2-associated death protein [90,91]. They can also activate RAS kinase, which is responsible for the activation of MAPK kinase (MEK) and downstream extracellular signal-regulated kinases (ERK). In other words, under hypoxia conditions, growth factors activate intracellular PI3K pathway promoting cell proliferation. Moreover, hypoxia is related to transcription factors, and like Twist, implicated in the cell

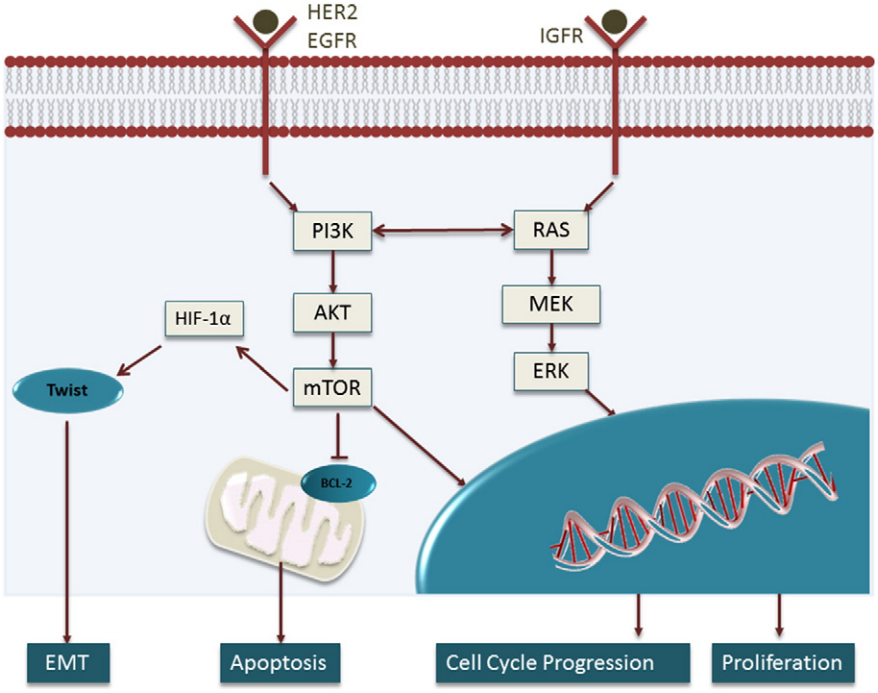

Fig. 2. Schematic representation of key signaling pathways involved in tumorigenesis. The cell membrane receptor tyrosine kinase human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR) and insulin growth factor receptor (IGFR) are activated mainly by binding of their ligands, which leads to activation of phosphatidylinositol 3-kinase (PI3K)- and RAS-mediated intracellular pathways, as well as to their interplay. Among the Akt multiple targets is mammalian target of rapamycin (mTOR), which promotes cell cycle progression and inhibits pro-apoptotic members of the BCL-2 family. PI3K-mTOR axis activation induces hypoxia inducible factor-1α (HIF-1α) upregulation and from this and the consequent Twist activation all the information goes towards cell epithelial–mesenchymal transition (EMT). Activation of RAS kinase elicits downstream activation of mitogen-activated protein kinase (MAPK) kinase (MEK) and subsequently of extracellular signal-regulated kinases (ERK), which further result in cell cycle progression and proliferation.

EMT program. During tumor progression and metastasization, PI3K pathway via mTOR upregulates HIF-1α leading to activation of Twist and consequently EMT [92–95]. Nevertheless, PI3K/Akt and RAS/MAPK have dependent pathways, leading to a complex and significant crosstalk [96,97].

Since PI3K pathway is activated by HER2, its relation to the resistance mechanism and tumor progression has been highlighted. Hyperactivation of PI3K pathway is also a consequence of *PIK3CA* gene that encodes for a catalytic subunit of PI3K. It can also result from mutations of PTEN, a tumor suppressor gene that can inhibit Akt action [98,99], although this alteration is not very common in BC patients [100,101]. For the major types of BC, the correlation between overexpression of ER and PR with PI3K pathway is not completely clear, but there are evidences that PI3K is important in ER signaling. Usually, hormonal-dependent BC develops endocrine resistance, promoted by decrease of ER, deregulation of its pathway and increase of PI3K signal, suggesting a crosstalk between ER and growth factor receptor pathway [102,103].

Another critical feature associated with the proliferative phenotype of cancer cells is related to an increased requirement for lipids, especially cholesterol due to their importance in membrane constitution and stabilization. In fact, cholesterol plays an important role in proliferative and apoptotic signaling pathways, such as PI3K/Akt, MAPK/ERK, HIF-1α and p53, being also the precursor of important hormones, such as estrogen and progesterone [104–106]. Consequently, an increased expression of LDL-cholesterol receptor is found in BC cells and there are evidences that support a correlation between LDL with cell division and loss of adhesion and migration [106,107]. Also related to signal transductions are the lipid rafts, important membrane structures rich in cholesterol and increasingly associated with tumor invasion and dissemination in BC [108,109].

Finally, the impact of the apoptotic process failure is important. In physiological conditions, p53 that initiates the apoptotic pathway through the interaction with BCL-2 family members in mitochondria is stabilized and negatively regulated by the mouse double minute 2 protein. However, in pathological conditions, this protein releases p53 that is activated as a transcriptional factor, leading to modulation of genes responsible for cell cycle arrest and apoptosis. p53 negative BC cells, produce an abnormal protein that is incapable of binding to target genes. Consequently, cells with p53 mutations have deregulated apoptotic pathways thus supporting cell survival and tumor proliferation [110,111].

Despite convincing evidence of the importance of the herein discussed intratumoral mechanisms in tumor genesis, other cell features have a major impact on cell survival. A large number of biological processes at molecular level are associated with drug sequestration and excretion. Generally, changes in membrane composition such as the overexpression of efflux transporters are responsible for drug sequestration and decreased drug uptake resulting in a reduction of intracellular drug concentration allowing the cell to become chemoresistant [112]. The question that decisively arises is: how does malignancy affect cell survival under chemotherapy? In the next section, structure, the physiologic and pathologic roles of these efflux transporters will be discussed.

3. The impact of efflux transporters in cancer therapeutic outcomes

Multidrug resistance is a phenotype exhibited by malignant cells characterized by resistance to multiple cytotoxic drugs with different structures, targets and mechanisms of action. This phenomenon can be classified as acquired or intrinsic: in intrinsic MDR, cancer cells already reveal resistance to drugs at initial exposure to treatment, while in acquired MDR the resistance is gained during chemotherapy. In the last section, many biologic aspects important to MDR have been discussed; here, we will focus on efflux transporters whose overexpression is extremely relevant in MDR, contributing to increased interest and research efforts in order to understand the mechanisms involved and to find molecules able to inhibit the efflux transport as a strategy to improve the efficacy of cancer treatment.

The efflux transporters are members of a superfamily of proteins that possesses an ATP-binding cassette (ABC), also known as nucleotide binding domain (NBD) [113]. There are 49 genes, arranged in seven subfamilies (termed A–G) encoding the transporters of this family. Many of the members are related to MDR [114,115]. Among them are the subfamilies B, C and G that encode for P-gp (ABCB1), MRPs (ABCC1-9) and BCRP (ABCG2), which have been the most studied and have the greatest relevance in cancer treatment. The subfamily B has even been called the MDR family of ABC transporters due to such relevance in cancer cells [113].

The efflux transporters are expressed in organs such as lungs, brain microvasculature that forms the blood–brain barrier (BBB), testis and placenta [116–118], where they play an important role in organ protection in physiological conditions, by pumping out from the cell a variety of structurally diverse metabolites and their conjugates [116]. However, they also pump out from the cell several xenobiotics [116], promoting a lower intracellular concentration of important drugs for cancer treatment, therefore precluding the therapeutic effect.

Importantly, overexpression of efflux transporters in CSCs is the principal mechanism of MDR [119,120]. In accord with the tumor environment, CSCs are exposed to pressures that promote overexpression of drug efflux transporters and, consequently, intrinsic MDR. Under certain stimuli, such as HIF-1α and IL-6, activation of *ABC* genes occurs, increasing the expression of ABC transporters and causing drug resistance [121,122].

The primary localization of these transporters is the plasma membrane where the substrates are exported in an ATP-dependent manner [116]. This active transport can occur against high concentration gradients, where ATP hydrolysis at their NBDs is the source of energy [123]. The main features of P-gp, MRP and BCRP in cancer perspective will be reviewed in the following sections. Their structure and cellular localization are schematically presented in Fig. 3, and some of their inhibitors, mechanism of action and pharmacologic compounds are summarized in Table 1.

3.1. P-glycoprotein

P-gp, originally isolated from Chinese hamster ovary cells exhibiting the MDR phenotype [124], was the first ABC transporter identified and is the most studied drug efflux transporter. P-gp, also known as MDR1 or PGY1 [116], is a 170 kDa protein with 1280 amino acids. It is constituted by two transmembrane domains (TMDs), each consisting of six transmembrane segments, two NBDs, and with intracellular N- and C-termini (Fig. 2). The two TMDs are hydrophobic, while the two NBD are hydrophilic, intracellular and ATP binding domains [116]. The first extracellular loop is N-glycosylated, an important aspect that routes and stabilizes membrane insertion [125]. The human structure shares 87% homology with mice in a drug binding state, important for in vivo studies of this protein and inhibitors [126]. This protein is primarily located in the apical membrane of epithelial and endothelial cells where it plays an important physiological role in the protection of the organ [116,127]. Its overexpression has been observed in several pathological conditions [128,129], including cancer [130,131]. However, the overexpression of P-gp is associated with MDR and ever since many important drugs have been identified as substrates of this efflux transporter [132–134].

The mechanism underlying the transport of substances is not completely clear, but there is evidence that substances are inserted into the inner hemileaflet of cell membranes before being expelled to the extracellular environment [203]. The unique common property of all the great numbers of P-gp substrates is their amphiphilic nature. Nonetheless, there are other structural properties that can be identified in substances transported by this protein, such as organic molecules

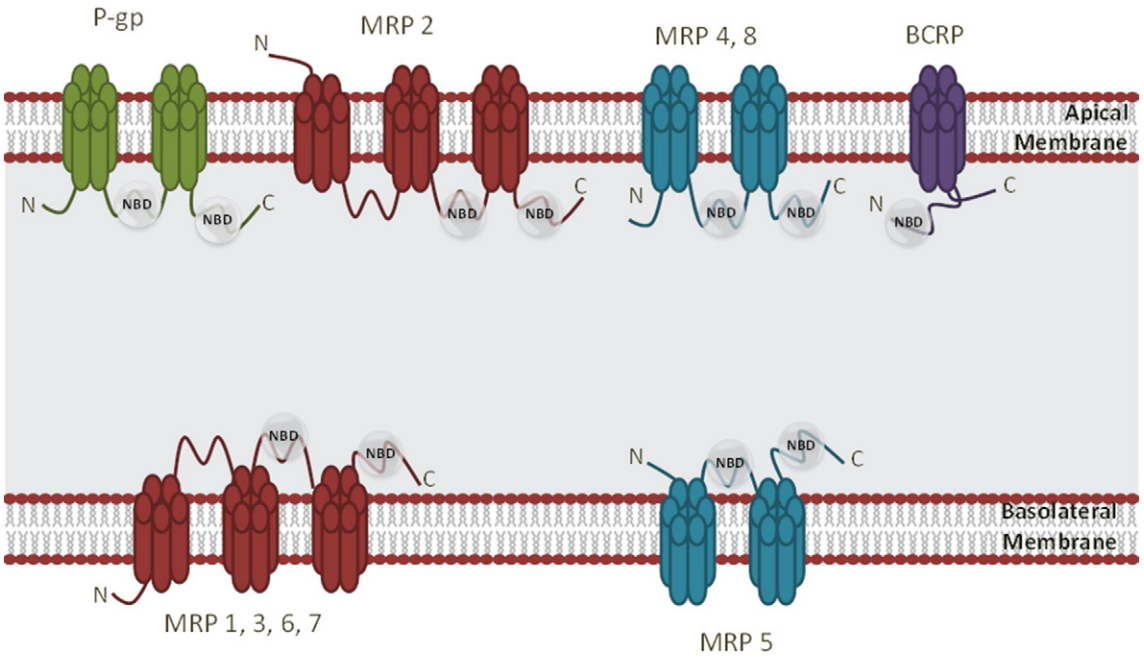

Fig. 3. Schematic representation of the localization and structure of P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP) 1–8, and breast cancer resistance protein (BCRP). P-gp is mainly localized at the apical membrane and is constituted by two nucleotide binding domains (NBD) and two transmembrane domains, each consisting of six transmembrane segments. MRP2, 4 and 8 are localized at the apical membrane, MRP1, 3, 6 and 7 are at the basolateral membrane, while the localization of MRP9 is still not established. MRP1, 2, 3, 6, and 7 have three transmembrane domains (two of them with six segments and one with five segments) and two NBD domains. MRP4, 5, 8, and 9 having a constitution much similar to that of P-gp, presenting two transmembrane domains (each with six transmembrane fragments) and two NBDs. BCRP is localized at the apical membrane and formed by only one NBD and one transmembrane domain, consisting of six transmembrane fragments.

with 200 to 1900 Da with neutral or weakly basic nature, or the presence of aromatic groups. Due to the fact that P-gp substrates are moderately hydrophobic, in the absence of active transport, they can cross membranes by passive diffusion. It means that if the passive diffusion is larger than the P-gp active transport, the compound will accumulate in cells and intracellular concentration should not be dependent on the P-gp transport.

Since the efflux transport protein becomes an important part of the tumor cell membrane, it can affect the activity of other proteins. For example, MDR cells are less responsive to caspase-dependent cell death and to complement-mediated cytotoxicity [204,205], consisting of another mechanism capable of promoting resistance. In BC, overexpression of P-gp is correlated with the most aggressive phenotype and poor outcomes, since proliferation and invasion mechanisms are active in chemoresistant cells [206–209]. Consequently, it is necessary to establish the mechanisms that promote an increased expression of P-gp through P-gp/MDR1 gene modulation. Several important pathways in cancer appear to be related to an enhanced MDR1 gene transcription, such as NF-kB, β-catenin and PI3K, reassuring the relevance of these pathways in cancer therapeutics [210–212]. Moreover, MDR1 gene modulators are possible targets in cancer therapeutics, such as frizzled family receptor 1 (FZD1), a component of Wnt/β-catenin pathway that upregulates P-gp expression [211]. Interaction of P-gp with other proteins, such as Anxa2, a phospholipid-binding protein, is also associated with MDR mechanisms, especially with cell proliferation and angiogenesis [213]. Apart from genetic mechanisms related to increased expression of P-gp, the existence of microparticles is also a promoter of chemoresistance. These small vesicles can transfer P-gp from resistant cells to drug-sensitive cells, in the intracellular environment, enhancing the number of chemoresistant cells. Moreover, microparticles are able to sequester drug molecules, which make them a target in order to modulate MDR [214,215].

As previously stated, the use of P-gp inhibitors (Table 1) is one of the possible approaches to antagonize its activity. Initially, drugs such as verapamil and cyclosporin A, identified as first generation P-gp inhibitors, were used with the aim of counteracting cancer cell resistance to chemotherapy. Additionally, P-gp inhibition can be considered in pharmacological optimization, due to their importance in drug absorption and metabolism. Therefore, through inhibition of P-gp it is possible to increase drug bioavailability in the breast, as well as in other organs such as the brain and intestine. Inhibitors of P-gp can act by blocking substrate binding sites, or by interfering with membrane fluidity or ATP hydrolysis [216]. These approaches, useful in different types of treatments, are not practical in cancer therapy, due to the complex therapeutic regimens necessary for cancer treatment.

3.2. Multidrug-resistance-associated proteins

Discovery of MRP1, in 1991, led to the identification of more proteins involved in MDR [217–219]. Presently, the MRP family is formed by thirteen members, nine of which are responsible for MDR (MRP1-9, encoded by *ABCC1-9* genes) [219]. MRPs are proteins of variable sizes, ranging from 100 KDa and 930 amino acids in MRP9 [220] to 190 kDa and 1531 amino acids in MRP1 [218]. In polarized cells, MRP1, 3, 5, 6 and 7 are expressed in the basolateral membrane domain, while MRP2, 4 and 8 are located to the apical side of epithelial cells (Fig. 2) [218,221,222]. However, some findings are contradictory, indicating that MRPs, like MRP4, might have different membrane localizations according to the tissues [223,224], whereas placement of MRP9 is not known yet.

Structurally, MRP1, 2, 3, 6, and 7 are very similar, as depicted in Fig. 2. They have three TMDs, two of them with six segments and one with only five segments, two NBDs, an extracellular N-terminal and an intracellular C-terminal [225]. On the other hand, MRP4, 5, 8, and 9 present two TMDs with six transmembrane fragments, two NBDs and both N- and C-terminals intracellularly [225], similar to P-gp.

MRP1 is an efflux transporter with affinity mainly to amphiphilic organic anionic molecules, such as methotrexate; however, non-anionic drugs conjugated with the anionic tripeptide glutathione can also be

Table 1
Clinically relevant data about the efflux transporters P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP) and breast cancer resistance protein (BCRP).

ABC transporter	Inhibitor	ABC transporters inhibition			Metabolism	Observation	Mechanism of action	MDR reversal in in vitro studies	Clinical trials	Reference
		P-gp	MRP	BCRP						
P-gp	Verapamil	x			CYP3A4	First generation calcium channel blocker	Competitive inhibitor	Doxorubicin, idarubicin, bortezomib in leukemia, Ewing's sarcoma myeloma, lung and breast cancer cell lines	Clinical use	[135–139]
	Cyclosporine A	x	x	x	CYP3A4	First generation, immunosuppressant drug	Competitive inhibitor	Docetaxel, daunorubicin in leukemia and hepatocellular carcinoma cell line	Phases I, II	[140–142]
	LY335979 (Zosuquidar)	x			CYP3A4	Third generation	ATPase activity inhibition	vinblastine, doxorubicin, etoposide, taxol, paclitaxel in leukemia and breast cancer cell lines	Phase III in leukemia	[143–145]
	XR9576 (Tariquidar)	x			Not CYP3A4	Third generation	ATPase activity inhibition	Paclitaxel, mitoxantrone, vinorelbine in ovarian cancer cell line in advanced breast cancer	Phase II in lung, ovarian, cervix and renal cancer	[146–148]
	PSC 833 (Valspodar)	x			CYP3A4	Second generation Non-immunosuppressive Cyclosporine A analog	Non competitive inhibitor	Doxorubicin, daunomycin, paclitaxel, vinblastin in leukemia, ovary cells and renal carcinoma cell lines	Phases I, II, III	[149–151]
	GF120918 (Elacridar)	x		x	Not CYP3A4	Third generation Orally active	ATPase activity inhibition	Topotecan in lung, breast cancer cell lines		[152–155]
	OC144-093	x				Third generation	ATPase activity inhibition	Doxorubicin, paclitaxel, and vinblastine lymphoma, breast, ovarian, uterine, and colorectal carcinoma cell lines		[156]
MRP	Myricetin		x			Flavonoid, found in plant antioxidants	Competitive inhibitor	Calcein, PhIP, vincristine in canine kidney cell lines, human colorectal adenocarcinoma		[157–161]
	MK571		x			Leukotriene LTD4 receptor antagonist	ATPase activity inhibition	Etoposide, NU/ICRF 505, mitoxantrone, colchicine in stomach, colon and breast cancer cell line		[162–167]
	Sulindac		x		CYP1A1	Nonsteroidal anti-inflammatory drug	Competitive inhibitor	Doxorubicin, epirubicin, Anthracycline, daunorubicin, rhodamine in lung, cervical, breast, melanoma carcinoma cell lines	Phase II in Leukemia Phase III in colon cancer	[168–174]
	Genistein		x		CYP1A2	Isoflavone, found in plants	Competitive inhibition	pirarubicin, calcein, daunomycin, daunorubicin in lung cancer, leukemia cell lines	Phase II in prostate, colon cancer	[175–181]
BCRP	Fumitremorgin C			x	CYP3A4	Fungal toxin used only in vitro	Competitive inhibitor	Mitoxantrone, doxorubicin, and topotecan in multiple myeloma and breast cancer cells lines		[182–184]
	Ko143			x		Fumitremorgin C analog	Competitive inhibitor	Topotecan, mitoxantrone in leukemia cell		[185–187]
	Gefitinib	x		x	CYP3A4	Epidermal growth factor receptor inhibitor	Competitive inhibitor	SN-38, irinotecan in breast, head and neck, prostate, breast, gastric, and colorectal tumors	Phase II in lung cancer	[188–191]
	HIV inhibitors (e.g., Ritonavir)	x	x	x	CYP3A, CYP2D6	Protease inhibitors		Doxorubicin, mitoxantrone in breast cancer and kidney cell lines	Phase I in solid tumors Phase II in pancreatic cancer	[192–194]
	Imatinib			x	CYP3A4	Tyrosine-kinase inhibitor	Competitive inhibitor	Topotecan, SN-38, mitoxantrone in breast cancer, embryonic kidney cell lines	Phase I in advanced cancers	[195–197]
	Flavonoids		x	x	Various CYP1A1	Found in plants		Mitoxantrone, SN-38 and bodipy-FL-prazosin in leukemia, breast and lung cancer		[196,198–202]

PhIP, 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine.

transported [226]. Although MRP1 and MRP2 drug substrates could be very similar, some important differences can be found, such as the affinity to the anticancer drug cisplatin [227].

Constitutive activation of the MRP members' expression is strongly related to some cancer relevant signaling pathways demonstrating the involvement of these membrane proteins in chemoresistance and tumorigenesis [219,228,229]. For instance, nuclear factor erythroid 2-related factor 2 can promote overexpression of MRP member 2, 3 and 4 through positive regulation of gene transcription, and mutations in this nuclear factor are related to increased MRP3 mRNA. There is also strong evidence that such nuclear factor is correlated with the angiogenic and metastasis mechanisms, constituting an interesting target in cancer therapeutics. In BC, specifically in ER and PR positive cancer, MRP8 is upregulated, contributing to chemoresistance, while overexpression of MRP3 has been recently related to HER enriched tumors [230–232].

3.3. Breast cancer resistance protein

BCRP is the most recently discovered ABC transporter that was firstly identified in MDR BC cell lines, and named as a result of it. This protein is also denominated mitoxantrone resistance protein and is encoded by *ABCG2* gene [116]. A number of publications suggest that it is expressed in the apical plasma membrane domain of both epithelial and endothelial cells (Fig. 2) [233,234]. Structurally unique in the ABC family, with only one TMD consisting of six transmembrane fragments, one NBD, and intracellular N- and C-termini, this protein can function as homo- or heterodimer with molecular mass ranging from 72 to 180 kDa [235].

As the other members of the ABC superfamily, BCRP is predominantly located within the plasma membrane, being dependent on membrane cholesterol composition. It is now acknowledged that BCRP interacts with caveolin-1, the main component of caveola membranes involved in transport and signaling processes. This protein–protein interaction is not well understood, but suggests a positive modulation by caveolin-1 on BCRP [236,237]. These structural aspects are relevant to comprehend the drug transport mechanisms and the environmental factors that modulate BCRP activity [236,238]. Interestingly, in BC there are evidences of BCRP presence, not only in the cell membrane, but also in cytoplasmic vesicles capable of drug sequestration, comprising a mechanism of drug compartmentalization that is an additional process in chemoresistance [239–241]. Moreover, structural and other types of proteins appear to have important roles in BCRP regulation. The transmembrane glycoprotein CD147 can upregulate BCRP expression, modulate function and localization of this drug efflux, while CK8, a cytoskeletal protein, is involved in drug resistant cells [242,243].

Currently, the importance of BCRP relies in its ability to transport a broad range of anticancer agents with important consequences in BC treatment. Among several therapeutic agents, tyrosine receptor inhibitors are now in the clinic, because of the importance of signaling cascades induced by numerous tyrosine receptors, such as PI3K and MAPK in the initiation, progression and metastasis of cancer. Interaction between tyrosine receptor inhibitors and BCRP compromises therapeutic effect and toxicity, mechanisms which also occur for P-gp [244,245]. Pro-inflammatory cytokines, such as IL-1β and TNF-α, which enhance BCRP mRNA, are also capable of increasing BCRP expression through NF-kB activation [246,247]. Among different pathways that influence BCRP expression, the pro-apoptotic p53 protein is responsible for negative control of BCRP expression. However, in many solid tumors, mutations of the *p53* gene inhibit the apoptotic mechanism contributing therefore to the absence of suppression on BCRP [246].

Several gene polymorphisms appear to have influence in cell BCRP expression although the relation with treatment responses is not established. For example, polymorphisms of the *p53* gene, CYP2D6 and MRP2 increase BCRP expression and, consequently, resistance to chemotherapy [248]. Moreover, there are other molecules from important pathways that act as promoters of BCRP expression, such as Pim Kinase and protein kinase C, increasing cancer cell resistance [249,250]. Increased expression of BCRP influences the outcome of therapeutic approaches in the HER2 BC [239]. Importance of BCRP in chemoresistance has been leading to an increased progress in functional and mechanistic knowledge, promoting emerging targets and strategies to overcome MDR.

4. Conclusion and future perspectives

Human cancer biology and cell heterogeneity are complex, thus presenting many different challenges. Development of therapeutic strategies capable of reducing deregulated processes such as angiogenesis, hypoxia, survival and EMT, while enhancing cancer cell death, is essential. More important, MDR that has been mainly associated with cell survival under chemotherapy is a major drawback in BC clinical outcomes. Disappointing results with P-gp inhibitors prior to chemotherapy highlight the relevance of a better understanding of the MDR promoting events in malignant cells thus contributing to the design of new therapeutic strategies able to enhance drug antitumor effect while avoiding MDR-related signaling pathways.

Nanomedicines have emerged as an interesting approach for in vivo delivery of a broad range of therapeutic agents. Their ability to circumvent biological barriers allows us to extrapolate that carrier-mediated transport for efficient drug intracellular targeting might represent a strategy to overcome MDR proteins. Moreover, nanocarriers dramatically change therapeutic entity pharmacodynamics, enhancing efficacy with minimal adverse effects, ensuring therefore that pharmacologic drug concentrations at the site of action would be achieved. Accordingly, we observed high intracellular drug levels upon breast cancer cell incubation with nanoincorporated paclitaxel in soild lipid nanoparticles. Conceptually, nanomedicines should be explored to modulate cancer cell pathways related to chemoresistance, particularly those involved in MDR proteins expression. Alternatively, nanomedicine tropism to undergo receptor or non-receptor endocytic internalization could be explored as a potential strategy to avoid substrate recognition by the membrane MDR proteins. At least hypothetically, the entrapped drug molecules entering the target cells by different mechanisms as compared to the free drug may evade efflux-transporters like P-gp, MRP and BCRP. Breast cancer eradication is anticipated once the anticancer drugs entrapped in the nanocarrier will not be recognized by the membrane efflux-transporters and consequently the receptors will not be involved/activated during the internalization.

Collectively, once the cell molecular targets are clearly defined and innovative strategies are developed, modern biomedicine will end breast cancer patients' tomorrow uncertainty.

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