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Review

Targets and mechanisms of berberine, a natural drug with potential to treat cancer with special focus on breast cancer

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ABSTRACT

Breast cancer is the most common cancer among women worldwide and novel therapeutic agents are needed to treat this disease. The plant-based alkaloid berberine has potential therapeutic applications for breast cancer, although a better understanding of the genes and cellular pathways regulated by this compound is needed to define the mechanism of its action in cancer treatment. In this review, the molecular targets of berberine in various cancers, particularly breast cancer, are discussed. Berberine was shown to be effective in inhibiting cell proliferation and promoting apoptosis in various cancerous cells. Some signaling pathways affected by berberine, including the MAP (mitogen-activated protein) kinase and Wnt/β-catenin pathways, are critical for reducing cellular migration and sensitivity to various growth factors. This review will discuss recent studies and consider the application of new prospective approaches based on microRNAs and other crucial regulators for use in future studies to define the action of berberine in cancer. The effects of berberine on cancer cell survival and proliferation are also outlined.

Contents

1. Introduction

Breast cancer is the most frequently diagnosed cancer in women, and is the second most common cancer worldwide. According to GLOBOCAN (2012), 25% (1.67 million) of all new cancer cases and 15% (522,000) of all cancer deaths in women were due to breast cancer (American Cancer Society, 2013).

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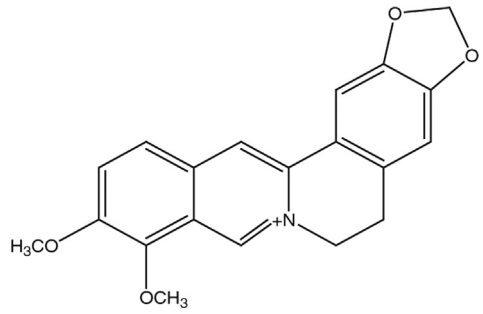

Fig. 1. Berberine structure.

Berberine can be isolated from the stems and roots of several plants, such as *Berberis vulgaris* and *Coptis chinensis* (Vuddanda et al., 2010; Bhardwaj and Kaushik, 2013; Potdar et al., 2012). Berberine (PubChem CID: 2353) is a nitrogenous cyclic compound (Fig. 1) with a structure that is highly similar to that of intercalating agents (e.g., ethidium) (Krey and Hahn, 1969; Bhardwaj and Kaushik, 2013). Intercalating agents are often used as nucleic acid dyes to study cell functions, and berberine is a well-known alkaloid drug that is commonly used as a fluorescent dye.

Berberine induces apoptosis and inhibits cell proliferation in various cell lines derived from breast, lung, colon, and liver cancer. However, berberine has been shown to have synergistic effects on cells treated in combination with more toxic drugs, including vincristine and irinotecan (Wang et al., 2014; Yu et al., 2014). Previous studies showed that the toxicity of vincristine towards hepatoma cells was reduced by combinatorial effects of berberine (Wang et al., 2014), and cell resistance to drugs was decreased by combination treatments with berberine (Yu et al., 2014).

Despite these findings, berberine efficacy and the molecular regulators that are targeted by berberine remain unclear. To date, literature on molecular properties and anti-cancer effects of berberine with special focus on breast cancer is scarce and a thorough review on the topic has not been done. Most of the current literature on berberine as the anti-cancer agent has been based on pharmacognosy and clinical evident. This review will aim to provide an extensive analysis of berberine effects on various molecular mechanisms (e.g. tumor suppressor genes, oncogenes and other regulators such as microRNAs, and berberine–nucleic acid interaction) involved in anti/oxidation, apoptosis and various signal transduction pathways discussed in relation to breast cancer development and therapy. In addition, this review also covers the metabolism, toxicity and adverse effects of berberine.

2. Bio-molecular activity of berberine

2.1. Antioxidant/oxidant activity of berberine

Free radicals, oxidative stress, and radiation-induced DNA damage, including oxidation, strand breakage, and ionization may lead to defects in genes involved in proliferation and cell signaling pathways that are crucial for tumor growth and cancer progression. Hence, antioxidants play a protective role in preventing cellular damage due to oxidation.

Several *Berberis* species, such as *B. cretica* (whole body) (Kukula-Koch et al., 2013), *B. microphylla* (fruit) (Ruiz et al., 2010), *B. koreana* (bark) (Qadir et al., 2009), *B. aristata* (root) (Singh and Kakkar, 2009) and *B. croatica* (root, twigs, and leaves) (Zovko Koncić et al., 2010) were shown to have anti-oxidant activity that can be attributed to a high phenolic content. Interestingly, about 60% of *Berberis* root extracts was shown to consist of berberine (Abd El-Wahab et al., 2013). Berberine has been demonstrated to be the compound that exerts antioxidant activity not only in MCF-7, HepG-2, and CACO-2 cancer cell lines but also in the normal PBMC cell line (Tomosaka et al., 2008; Abd El-Wahab et al., 2013).

Reactive oxygen species are mainly oxidative byproducts of cells that can be neutralized by specific enzymes or antioxidant agents. The three main enzymes that generate cellular Reactive oxygen species are lipoxygenase (Vavreckova et al., 1996), xanthine oxidase (Kim et al., 1998), and cyclooxygenase-2 (COX2) (Lin et al., 2002; Liu et al., 2013a). Reduced activation of these enzymes in turn leads to reduced levels of Reactive oxygen species. Nevertheless, antioxidant activity of other alkaloids such as sanguinarine and chelerythrine isolated from *Chelidonium majus* can inhibit lipoxygenase (Vavreckova et al., 1996), while berberine was effective in reducing the activity of xanthine oxidase (Chang et al., 1994) and COX2 (Lin et al., 2002; Liu et al., 2013b), which also decreases Reactive oxygen species levels. In addition, berberine has been shown to decrease the activity of superoxide dismutase and subsequently reduce Reactive oxygen species levels (Liu et al., 2008).

In contrast to the antioxidant activity of berberine discussed earlier, many studies have strongly suggested that berberine increased the amount of Reactive oxygen species to induce several apoptotic signaling pathways, including MAPK (mitogen-activated protein kinase), ERK1/2 (Extracellular signal-regulated kinase 1/2), JNK (c-Jun N-terminal protein kinase) and Akt, as well as calcium dependent pathways (Jantova et al., 2007; Lin et al., 2006; Meeran et al., 2008). However, berberine has been shown to have different effects on cellular Reactive oxygen species content depending on cell conditions and types. For example, berberine induces Reactive oxygen species production in prostate cancer cells, but not normal epithelial cells (Liu et al., 2008; Meeran et al., 2008; Oberoi-Khanuja et al., 2013).

A better definition of the anti-oxidant activity of berberine in cancer cells still requires further study. Knowledge of the dose-dependent action of berberine would be advantageous for the use of this compound as a chemoprotective and chemotherapeutic agent in normal and affected cells, respectively. Some strong antioxidant agents such as NAC (N-acetyl-L-cysteine) have been shown to reduce Reactive oxygen species levels and remarkably decrease berberine-induced apoptosis, likely by preventing berberine-induced release of cytochrome c and Smac/DIABLO, an apoptotic protein (Oberoi-Khanuja et al., 2013; Hsu et al., 2007). Interestingly, previous studies suggested that strong antioxidants may protect cancer cell from apoptosis, and thus promote cell survival.

2.2. Berberine and apoptosis

Apoptosis is regulated by different molecules that lead to cell death induced by mitochondrial collapse, caspase activation and subsequent DNA fragmentation. Some membranous proteins including Fas (death receptor) and FasL (Fas ligand), and increased cytoplasmic levels of proteins such as Smac/DIABLO, Bax (Bcl-2-associated X protein) and cytochrome c are also involved in the activation of apoptosis. On the other hand, cells also express apoptosis inhibitors, including Bcl-2, IAP (Inhibitor of Apoptosis Protein), and XIAP (X-linked inhibitor of apoptosis protein) (Oberoi-Khanuja et al., 2013).

Apoptosis involves various signaling pathways. Most important apoptotic pathways are (1) Reactive oxygen species - dependent apoptosis, (2) Fas-dependent apoptosis, (3) p53-dependent apoptosis and (4) p53 independent apoptosis. Berberine has been shown to affect all of these processes (Piyanuch et al., 2007; James et al., 2011; Hsu et al., 2007).

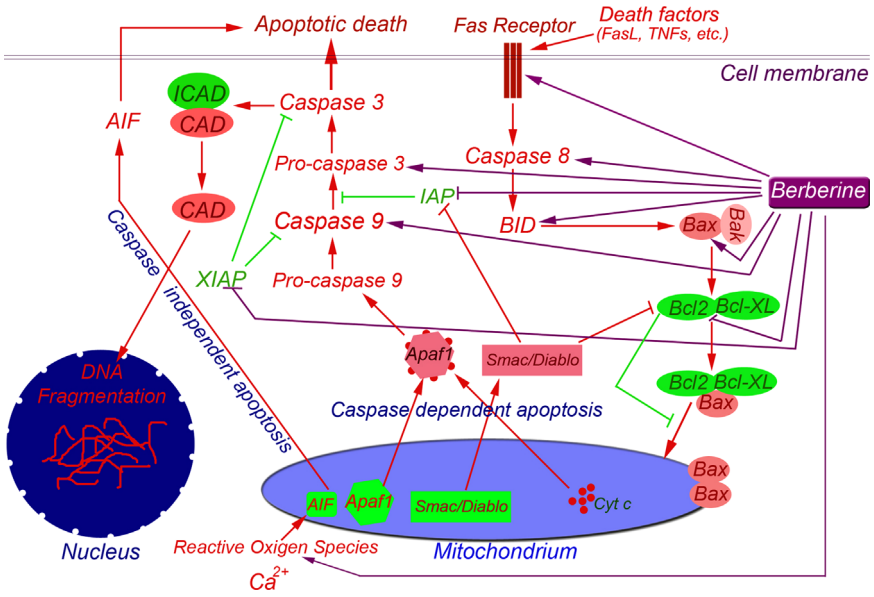

Fig. 2. Simplified apoptotic pathway. Apoptosis is induced by an apoptotic signal, such as that from virus-infected cells that present Fas ligand on the cell surface, and/or some immunological compounds, including tumor necrotic factor α (TNF-α). Some apoptosis inhibitors normally promote cell survival. After attachment of Fas receptor (FasR) and Fas ligand (FasL), apoptosis is triggered by caspase-8 activation, followed by Bax activation and in turn Bcl-2 deactivation. This process is continued by Bax-induced pore formation in the mitochondrial membrane, which allows cytochrome c, Smac/DIABLO, and Apaf-1 release from the mitochondria and subsequent activation of a sequential caspase cascade, and finally DNA fragmentation induced by caspase-activated DNAase (CAD). ICAD is Inhibitor of CAD. Apoptosis inducing factor (AIF) is another molecule that directly responds to Reactive oxygen species and the mitochondrial calcium concentration. Berberine induces apoptosis by decreasing levels of the apoptosis inhibitors (IAP, XIAP, and Bcl-2), and increasing amounts of apoptosis activators (caspase-3, caspase-8, caspase-9, Bax, Bid and IAF).

Berberine induces apoptosis by increasing the level of Reactive oxygen species and some Reactive oxygen species-associated signaling pathways such as JNK/p38 MAPK (Hsu et al., 2007), the calcium-dependent protein kinase protein kinase C (PKC), ERK, and glycogen synthase kinase-3β (Piyanuch et al., 2007). Furthermore, berberine was also reported to be involved in caspase-independent apoptosis, which is directly activated by Reactive oxygen species generation (see previous section). The higher Reactive oxygen species levels can alter the mitochondrial membrane potential, leading to mitochondrial collapse. As a consequence of Reactive oxygen species production, activation of AIF (apoptosis-inducing factor), a protein involved in caspase-independent apoptosis, occurs to induce apoptosis (Murahashi et al., 2003).

In addition to Reactive oxygen species-dependent apoptosis, berberine has been shown to significantly activate certain caspases such as caspase-3, caspase-8 (Ho et al., 2009), and caspase-9 (Lin et al., 2006; Ho et al., 2009; Mantena et al., 2006). Caspases are proteolytic enzymes that are activated by apoptotic factors, including FasL and tumor necrotic factors, and target cellular enzymes. Berberine can induce expression of both Fas and FasL in cancer cell lines to induce caspase activity (Hwang et al., 2006; Hsu et al., 2007). Moreover, berberine can activate apoptosis by deactivating two major caspase inhibitors, namely c-IAP1 and XIAP, which also leads to apoptosis activation (Lin et al., 2002; Hsu et al., 2007).

In addition to caspases, p53 (tumor protein p53) is a tumor suppressor protein that mainly controls cellular homeostasis. Berberine can activate p53 gene expression and phosphorylation, leading to apoptosis and cell cycle arrest (Lin et al., 2006; Liang et al., 2008). In neuroblastoma cells, berberine inhibited p53-dependent cell growth by apoptosis induction (Choi et al., 2008, 2009). Cell cycle suppression and apoptosis induction is orchestrated by p53 acting as a tumor suppressor protein. p53 can produce and increase levels of another tumor suppressor protein, Cip1/p21 (cyclin-dependent kinase inhibitor 1A), as well as other proteins involved in apoptosis activation such as Bax, Puma, Noxa, Apaf-1 (*Apoptotic protease activating factor* 1), and Fas receptor (Xiong et al., 1993; Vousden and Lu, 2002). p53 can also transcriptionally inhibit the expression of anti-apoptotic proteins such as Bcl-2, Bcl-xL, and survivin (Hoffman et al., 2002).

Bax is another apoptotic protein that promotes cytochrome c release by pore formation in mitochondrial membranes (Fig. 2). Berberine increased Bax levels to affect the Bax/Bcl-2 ratio such that it favors apoptosis (Lin et al., 2006; Mantena et al., 2006; Hwang et al., 2006; Hsu et al., 2007; Ho et al., 2009). Berberine can also decrease the expression of anti-apoptotic genes such as Bcl-2, Bid, and Bcl-xL (Ho et al., 2009; Eom et al., 2008). In cells lines, apoptosis induced by berberine can either be dose-independent or dose-dependent (He et al., 2012). In a cellular model of cardiac injury, berberine reduced apoptosis in doxorubicin-treated cardiomyocytes, which also showed decreased levels of caspase-3 and caspase-9 activity (Lv et al., 2012). Therefore, cell conditions may affect the role of berberine, indicating that further investigation into associated antioxidant activities of berberine is needed.

Berberine has been shown to induce apoptosis in two breast cancer cell lines, MCF-7 and MDA-MB-231 (Kim et al., 2010; Patil et al., 2010), through a mitochondria-dependent pathway that involves increased levels of cytoplasmic cytochrome c, caspase-9 activity and poly (ADP-ribose) polymerase (PARP) cleavage as well as decreased amounts of Bcl-2. In addition, immunoblotting results demonstrated that p53 and Kip1/p27 (Cyclin-dependent kinase inhibitor 1B) expression was up-regulated by berberine, which suggests that this compound has pro-apoptotic properties in cancer cells (Patil et al., 2010). The targets of berberine are shown in Tables 1 and 2.

2.3. Berberine and nucleic acid interaction

The interaction between berberine and DNA is partly responsible for its anti-cancer activity. Previous studies showed that berberine can directly bind to DNA in a pH-dependent manner to form a DNA-berberine complex (Krey and Hahn, 1969; Rungsitiyakorn et al., 1981). Polyadenylic acid [poly (A)] binds more strongly to berberine than do other polynucleotides such as poly (U) and poly (C) (Creasey, 1979; Islam and Suresh Kumar, 2008).

Table 1
Molecules directly associated with cancer that have increased expression following berberine exposure. Possible clinical applications of berberine treatment are specified.

Molecules	Effect of berberine	Biological results	Application	References
Reactive oxygen species	Increased	Cathepsin B release Apoptosis Inhibitory Factor (AIF) release Apoptosis induction	Prostate cancer Colon cancer	Oberoi-Khanuja et al., 2013;Wang et al., 2012b
p53 [a]	Increased	Cip1/p21 transcription Cyclin D inhibition Cell division arrest Apoptosis induction	Lung cancer Gastric carcinoma Neuroblastoma Cervical cancer Breast cancer	Lin et al., 2006; Liang et al., 2008; Choi et al., 2008; Choi et al., 2009; Patil et al., 2010; Lu et al., 2010; Kim et al., 2012
Rb	Increased	Cyclin transcription blocking Cell division arrest	Cervical cancer	Mahata et al., 2011
ATM	Increased	Apoptosis induction	Prostate cancer	Wang et al., 2012a
Caspase 3 [a]	Increased	Apoptosis induction	Tongue squamous cancer Breast cancer Cervical cancer Liver cancer	Ho et al., 2009; Kim et al., 2010; Patil et al., 2010; Lu et al., 2010 ; Wang et al., 2014
Caspase 8[a]	Increased	Apoptosis induction	Promonocytic U937 cells Epidermoid carcinoma Prostate cancer Tongue squamous cancer Breast cancer	Oberoi-Khanuja et al., 2013; Ho et al., 2009; Kim et al., 2010; Patil et al., 2010; Lu et al., 2010; Letasiová et al., 2006
Caspase 9 [a]	Increased		Cervical cancer	
Fas receptor/FasL	Increased	Apoptosis induction	Liver cancer Colon cancer Cervical cancer Oral cancer Cardiomyocyte	Hwang et al., 2006; Lin et al., 2007; Hsu et al., 2007; Lu et al., 2010; Lv et al., 2012
Bax	Increased	Apoptosis induction	Tongue squamous cancer Epidermoid carcinoma Gasteric carcinomaColon cancer Liver cancer	Lin et al. , 2006; Mantena et al., 2006; Hwang et al., 2006; Hsu et al., 2007; Ho et al., 2009; Wang et al., 2014
BID	Increased	Apoptosis induction	Glioblastoma Tongue squamous cancer	Eom et al., 2008; Ho et al., 2009
IFN-β (Interferon-β) [a]	Increased	Chemosensitivity	Breast cancer	Liu et al., 2009a;
TNF-alpha	Increased	Apoptosis induction	Cervical cancer	Lu et al., 2010
Cip1/p21 [a]	Increased	G1 Cyclin inhibition Cell division arrest	Breast cancer Liver cancer Mesangial cells	Liu et al., 2009a; Patil et al., 2010; Lan et al., 2014; Wang et al., 2014
Kip1/p27 [a]	Increased	G1 Cyclin inhibition Cell division arrest	Breast cancer Mesangial cells	Patil et al., 2010; Lan et al., 2014
Wee1	Increased	G2/M phase arrest	Gastric carcinoma	Lin et al., 2006
ZO-1 [a]	Increased	Cell tight junction Cell division arrest Metastasis inhibition	Breast cancer	Liu et al., 2009a
E-cadeherin	Increased	Metastasis inhibition	Lung cancer	Qi et al., 2014
Cyp1A1 [a]	Increased	Estrogen reduction	Breast cancer	Wen et al., 2014
Cyp1B1 [a]	Increased	Estrogen reduction	Breast cancer	Wen et al., 2014
Ubiquitin ligase	Increased	Inhibit cell proliferation	Colon cancer	Wang et al., 2012a
miR-21-p	Increased	Growth suppression Apoptosis induction	Liver cancer	Lo et al., 2013

[a] Molecules investigated in breast cancer.

Consequently, berberine as an anti-sense agent may also bind to mRNAs to inhibit mRNA translocation and even translation. Berberine is a fluorescent compound, which allows its detection by various spectroscopic techniques such as absorbance, fluorescence, nuclear magnetic resonance (NMR), and mass spectrometry (Islam and Suresh Kumar, 2008). Berberine absorbance shows peaks at 230, 267, 344, and 420 nm and a peak emission of 550 nm (Serafim et al., 2008). Compared to ethidium, which is a strong intercalator, berberine has been demonstrated to partially intercalate to transfer RNA for phenylalanine (tRNAphe) (Islam et al., 2007, 2008). Berberine as an intercalator may cause DNA double-strand breaks that induce p53 and ATM (Ataxia Telangiectasia Mutated) activation and eventually causes apoptosis (Liu et al., 2009a), although the destructive effects of berberine on normal cells are not fully known and require additional investigation.

2.4. Berberine and cell signaling pathways

A membranous protein affected by berberine, EGF (epidermal growth factor) receptor, is a crucial biomarker of breast cancer. The EGF receptor ligand, EGF is a major compound that triggers cell proliferation. In colonic tumor cell lines EGF receptor levels are increased, while a special type of EGF receptor known as Her2 (Human Epidermal Growth Factor Receptor 2) is mutated in many breast cancer cases (Huang and Davidson, 2006). EGF receptor activation is suppressible, and berberine has been shown to downregulate EGF receptor gene expression (Wang et al., 2013). According to a study by Wang et al. (2013), berberine activates ubiquitin ligase, which leads to proteasome-mediated EGF receptor degradation (Liu et al., 2009b). In addition, expression of EGF receptor and HER2 in MCF-7 cells was shown to be upregulated by

Table 2
Molecules directly associated with cancer that have decreased levels in response to berberine exposure. Possible clinical applications of berberine treatment are specified.

Molecules	Effect of berberine	Biological results	Application	References
NF-κB [a]	Decreased	Chemosensitivity Cell cycle arrest	Breast cancer Lung cancer Colon cancer Prostate cancer Mesangial cells	Liu et al., 2013a; Wang et al., 2012a; Chidambara Murthy et al., 2012;Kuo et al., 2012; Lan et al., 2014
EGF	Decreased	Cell growth reduction Cell cycle arrest	Colon cancer	Wang et al., 2012a
Rho kinase	Decreased	Metastasis reduction	Nasopharyngeal cancer	Tang et al., 2009
c-IAP1	Decreased	Apoptosis induction	Colon cancer	Hsu et al., 2007; Yu et al., 2014
PDGF (Platelet-Derived Growth Factor)	Decreased	Cell growth reduction Cell cycle arrest	Vascular smooth muscle	Liang et al., 2008
TGF-β	Decreased	Metastasis inhibition	Lung cancer Mesangial cells	Lan et al., 2014; Qi et al., 2014
Raf/MEK/ERK	Decreased	Cell growth reduction Cell cycle arrest	Vascular smooth muscle Lung cancer	Liang et al., 2006; Liang et al., 2008; Fu et al., 2013
AP-1 [a]	Decreased	Cell growth reduction Cell cycle arrest	Cervical cancer Bladder cancer Breast cancer Mesangial cells	Kim et al., 2008; Mahata et al., 2011; Yan et al., 2011; Kuo et al., 2012; Lan et al., 2014
AP-2	Decreased	Switching off signaling genes Cell growth inhibition Cell cycle arrest	Lung cancer	Liu et al., 2013a
PI3K/Akt [a]	Decreased	Metastasis reduction Cell growth inhibition Cell cycle arrest	Lung cancer Breast cancer	Kuo et al., 2012; Liu et al., 2013a
CD147	Decreased	Cell cycle arrest	Liver cancer	Hou et al., 2011
XIAP	Decreased	Apoptosis induction	Leukemia	Lin et al., 2002
Wnt/β-catenin	Decreased	Metastasis reduction	Colon cancer	Park et al., 2012; Albring et al., 2013
VEGF receptor 2	Decreased	Cell division reduction	Leukemia	Lin et al., 2012
Her2 [a]	Decreased	Chemosensitivity Cell growth reduction Cell cycle arrest	Breast cancer	Lin et al., 2002; Pierpaoli et al., 2013
Bcl2 [a]	Decreased	Apoptosis induction	Gastric carcinoma Breast cancer	Lin et al., 2002; Lin et al., 2006
Bcl-x	Decreased	Apoptosis induction	Colon cancer	Yu et al., 2014
Survivin	Decreased	Apoptosis induction	Colon cancer	Yu et al., 2014
Cox2 [a]	Decreased	Chemosensitivity Cell cycle arrest	Breast cancer Lung cancer	Lin et al., 2002; Liu et al., 2013a
Cyclin-D1 [a]	Decreased	Cell cycle arrest	Breast cancer	Lin et al., 2002
Cyclin-B1	Decreased	Cell cycle arrest	Gastric carcinoma Colorectal cancer	Lin et al., 2006 ; Cai et al., 2014
CDK1	Decreased	Cell cycle arrest	Gastric carcinoma	Chang et al., 1994
Cdc2	Decreased	Cell cycle arrest	Colorectal cancer	Lu et al., 2010
Cdc25	Decreased	Cell cycle arrest	Gastric carcinoma Colorectal cancer	Chang et al., 1994; Lu et al., 2010
Rev3	Decreased	DNA synthesis blocking	chicken B lymphocyte	Hu et al., 2014
hTERT	Decreased	Telomerase inhibition Cell growth inhibition DNA instability	Cervical cancer Lung cancer	Mahata et al., 2011; Liu et al., 2013
TOPO1	Decreased	DNA synthesis blocking	Anti cancer	Qin et al., 2007
H-Ras	Decreased	Cell signaling arrest	Bladder cancer	Yan et al., 2011
miR-21	Decreased	Chemosensitivity Apoptosis	Ovarian cancer Myeloma cells	Hu et al., 2013; Liu et al., 2013a

[a] Molecules investigated in breast cancer.

berberine (Liu et al., 2009b). Meanwhile, berberine can also inhibit the expression of CD147, another integral membrane protein that interacts with cytoplasmic signaling molecules (Hou et al., 2011). Such decreases in CD147 levels affect the actions of external signals such as growth factors that induce cell growth (Yan et al., 2011). The accumulation of cyclin D depends on growth factors and deactivation of EGF receptor by berberine can inhibit this accumulation and in turn arrest the cell cycle. EGF also activates the serine-threonine kinase Akt and the mTOR (mammalian target of rapamycin), which are both essential for cell survival (Cao et al., 2009). Suppression of EGF by berberine may therefore indirectly affect AKT and mTOR leading to reduced cancer cell survival.

Similar to EGF receptor, vascular endothelial growth factor receptor 2 (VEGF receptor 2) is another membranous receptor that transmits external signals to cytoplasmic counterparts to induce cell proliferation. VEGF receptor is expressed in smooth muscle cells, macrophages, and endothelial cells, and plays a role in angiogenesis of cancer cells (Lin et al., 2012). Berberine also reportedly suppresses VEGF receptor 2 activation, which could indirectly decrease angiogenesis rates, although additional studies are needed to confirm whether angiogenesis is indeed affected by berberine treatment (Inoue et al., 1998; Lin et al., 2012).

PI3K/Akt (phosphoinositide 3-kinase/Akt) and MAP pathway (Raf/MEK/ERK) are two crucial cell signaling pathways and play

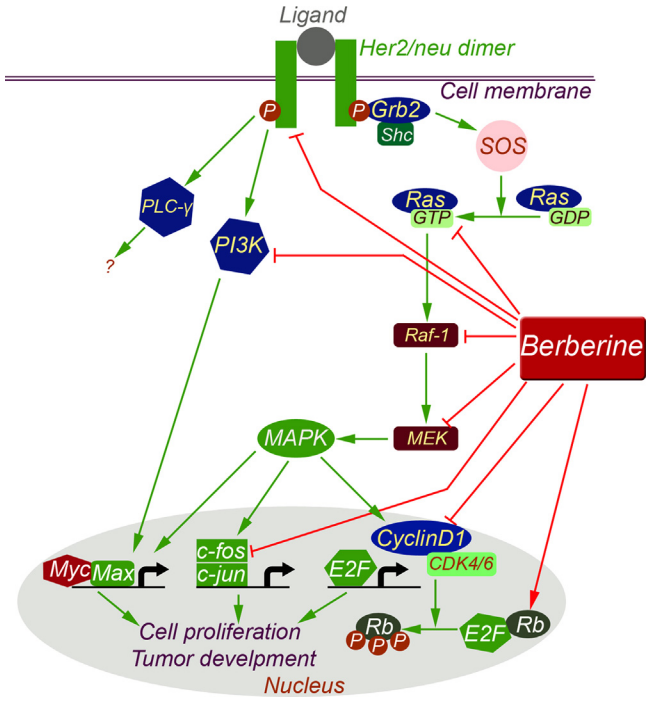

Fig. 3. Her2/neu signaling pathway. The her2/neu heterodimer is a growth factor receptor that activates cell proliferation. Mitogen-activated protein (MAP) kinase is another important signaling pathway. Upon ligand binding to the receptor, Ras is activated by GDP/GTP exchange that involves the guanine nucleotide exchange factor (GEF) SOS. Ras activation initiates a kinase cascade that results in phosphorylation of MAP, which then translocates to the nucleus to activate transcription of genes related to cell proliferation. One of the most important MAP targets is cyclinD1/ (cdk4/cdk6), which phosphorylates a tumor suppressor protein known as Retinoblastoma (Rb) that inhibits E2F transcription factor activity. When E2F is released, gene expression can occur. Activated enhancer-binding protein 1 (c-jun/c-fos) is another transcription factor activated by MAP. Berberine has been shown to decrease AP-1 and cyclinD1 levels, and also to affect MAP and PI3K pathways. This figure shows that berberine affects multiple points of signaling pathways, from the membranous receptors to the nucleus.

significant roles in regulating gene expression and cell proliferation. Both pathways are involved in complex cytoplasmic signaling networks, and are linked to membranous receptors such as EGF receptor and Her2, as well as nuclear transcription factors (Fig. 3). Several previous studies showed that berberine could inhibit Ras, Raf, MEK, and PI3K activities, which are critical proteins in various cell signaling pathways (Liang et al., 2006; Liang et al., 2008; Kuo et al., 2012; Liu et al., 2013a). The effects of berberine on nuclear aspects of these pathways are discussed in Section 2.5, with berberine being shown to strongly inhibit receptors, cytoplasmic signal transferring molecules, and gene activators to exert significant effects on signaling pathways.

Another signaling pathway affected by berberine involves Wnt/β-catenin. This pathway is closely related to cell adhesion, and its activation leads to migration and metastasis of cancerous cells. β-catenin is a membrane-linked protein involved in cell adhesion that upon activation dissociates from the membranous E-cadherin linked complex and translocates to the nucleus where it regulates transcription. Abnormal activation of Wnt/β-catenin signaling pathway can lead to colon cancer. Berberine has been shown to inhibit cell proliferation and migration by blocking Wnt/β-catenin signaling (Park et al., 2012; Albring et al., 2013) as well as increasing E-cadherin levels by down-regulation of transforming growth factor-β (TGF-β) (Qi et al., 2014). Metastasis is correlated with the reduced capacity of neighboring cells to attach to each other. Additional studies of signaling pathways associated with cell adhesion should help to define whether berberine can affect metastasis by regulating cell attachment activity. ZO-1 (Zonula Occludance-1) is an important molecule that is linked to the desmosomal complex and plays a major role in cell tight junctions, which helps adjacent cells to attach tightly to each other (Liu et al., 2009c). Activated ZO-1 attracts the cyclin-dependent kinase CDK4 from the nucleus, such that cells lacking nuclear CDK4 do not divide. Berberine can activate ZO-1, and therefore, could indirectly reduce cell mobility (Liu et al., 2009a), although further investigation is needed to clarify the effects of berberine on metastasis of various cancer types at a molecular level.

2.5. Berberine and cell proliferation

Inhibition of cell proliferation is closely associated with apoptosis. p53 activates transcription of many genes that mediate cell cycle arrest and apoptosis. When genetic errors accumulate in cells, their division is automatically stopped to allow repair of the defects, with apoptosis being initiated if the repair mechanisms do not work properly. Oral administration of berberine can inhibit both p53 expressing and p53 non-expressing lung tumor xenografts (James et al., 2011), as well as the G1 phase of cancerous cells by up-regulating p53 levels (James et al., 2011; Park et al., 2012). G1 inhibition also occurs when cyclin dependent kinase (CDK) inhibitors such as Cip1/p21 and Kip1/p27 are overexpressed. CDKs are heterodimeric proteins that promote the cell cycle by regulating kinase cascades. Cyclin D1 is a critical G1-cyclin that interacts with Cdk4 and Cdk6. Both Cip1/p21 and Kip1/p27 inhibit cyclin D1/cdk4 or cyclin D1/cdk6 heterodimers (James et al., 2011; Lan et al., 2014). Table 2 lists the various molecules that are targeted and suppressed by berberine.

Mutations in the p53 gene (TP53) are found in almost half of all cancer cases. One interesting study demonstrated that berberine in the breast cancer cell lines MCF-7 and MDA-MB231 had different effects on p53 expression. MCF-7 cells express wild-type TP53, while MDA-MB-231 cells have a mutated TP53. In both lines, p53 mRNA levels were decreased by TPA (12-O-tetradecanoylphorbol-13-acetate), a tumor promoter (Kim et al., 2012a). After berberine treatment, the level of p53 expression was increased in TPA-induced MCF-7 cells, but was not affected in TPA-treated MDA-MB-231 cells (Kim et al., 2012b). While studies showed that berberine does not directly affect mutated p53, it does suppress the cell cycle in G2 via p53-independent pathway when p53 is mutated (Wang et al., 2012a). Consequently, berberine can affect both p53-mutated and p53 non-mutated cancer cells by different pathways. In leukemia cells lacking p53, berberine could trigger apoptosis by down-regulating expression of the XIAP (X-linked inhibitor of apoptosis protein) (Lin et al., 2002).

ATM is another critical cell cycle protein that controls the G2 check point, and when DNA breakage is detected initiates DNA repair by homologous recombination (Smith et al., 2010). Berberine was shown to be a potential inhibitor of ATM (Liu et al., 2009a), and therefore apoptosis is likely initiated following ATM activation in berberine-treated cells due to inadequate DNA repair activity (Smith et al., 2010; Wang et al., 2012a). The role of berberine appears to be important for hereditary cases of breast cancer. Breast cancer proteins (BRCA1 and BRCA2) are two major mutated proteins that are related to hereditary breast cancer and are closely related to ATM (Huang and Davidson, 2006). Although the effects of berberine on BRCA1 and BRCA2 await evaluation, their relationship with ATM suggests that berberine may have similar increasing effects on these proteins (Fig. 4).

2.6. Berberine and gene expression

Some transcription factors and gene regulators are affected by berberine treatment. The nuclear factor *kappa* B (NF-κB) is activated by several inflammatory compounds and carcinogens,

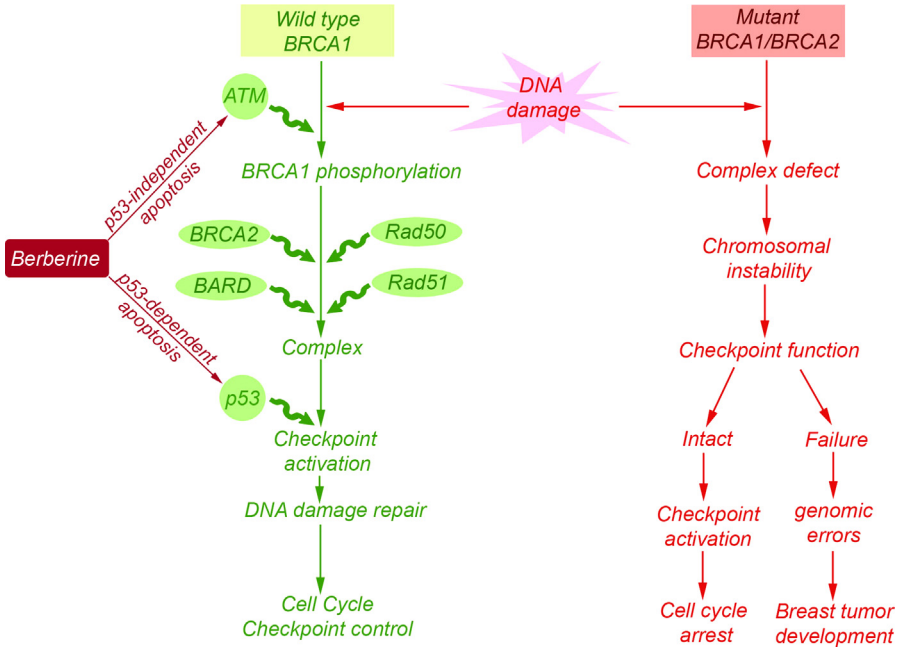

Fig. 4. BRCA1 and BRCA2 function in DNA repair in breast cancer cells. When DNA damage occurs, BRCA1 is phosphorylated by ATM. Mutated BRCA1 and BRCA2 cause checkpoint dysfunction that in turn leads to development of cancerous breast cells. Mutated ATM also affects normal DNA repair processes and can inhibit cell cycle arrest in response to DNA damage. Berberine is a crucial anti-cancer compound that affects ATM such that apoptosis is triggered via p53-independent pathway.

such as TNF-α, okadaic acid (OA), and cigarette smoke condensates, and is activated by phosphorylation and subsequent degradation of its inhibitor IκB. NF-κB (p65/p50) is widely expressed in cells with high rates of division (Liu et al., 2013a; Hu et al., 2013; Lan et al., 2014). NF-κB is normally inhibited by IκB (inhibitor of κB) in cytoplasm. Upon phosphorylation, IκB is released from NF-κB, which then translocates NF-κB to the nucleus to activate genes related to cell division and cell migration. Berberine down-regulates the expression of p65, an NF-κB monomer, and resulted in reduced levels of phosphorylated IκBα (Muralimanoharan et al., 2009; Chidambara Murthy et al., 2012; Liu et al., 2013a). Since NF-κB overexpression can promote cell migration, berberine-induced suppression of NF-κB expression may prevent metastasis (Liu et al., 2013a). Although further investigation is needed, the capacity of berberine to inhibit NF-κB levels in lung (Lee et al., 2007; Liu et al., 2013a), prostate (Muralimanoharan et al., 2009), colon (Chidambara Murthy et al., 2012), and breast cancer cells (Kuo et al., 2012) supports this possibility.

p53 can activate gene transcription of CDK inhibitors such as Cip1/p21 and Kip1/p27. As mentioned earlier, p53 can also be activated by berberine (James et al., 2011). Berberine has been shown to upregulate Cip1/p21 expression by more than 15-fold (Liu et al., 2009a). In cervical cancer, the expression of the human papilloma virus (HPV) proteins E6 and E7 were found to be reduced by berberine treatment. Berberine could also effectively increase p53 and Rb expression in HPV-positive cervical cancer cell lines, which may be effective in suppressing proliferation of cervical cancer cells (Mahata et al., 2011).

Human telomerase reverse transcriptase (hTERT) is a component of human telomerase, which synthesizes telomeric ends of chromosomes and maintains chromosome stability. Since continuous telomerase activity effectively confers cellular immortality, hTERT is typically not expressed in human somatic cells, although it is commonly overexpressed in various cancer types (Stewart et al., 2002). Activated enhancer-binding protein-2 (AP-2) has been shown to control hTERT expression at the level of transcription (Riechmann and Meyerowitz, 1998). By binding to the hTERT promoter, AP-2 factors can activate a number of cancer-related genes and signaling pathways, including hTERT, PI3K/Akt and Raf/MEK/ERK (Deng et al., 2007). The expression of AP-2 as well as hTERT can be reduced by berberine, and as a result, many signaling genes are switched off, which subsequently initiates apoptosis (Mahata et al., 2011; Kim et al., 2012a; Liu et al., 2013a).

Rev3 gene, which is a key player in translesion DNA synthesis, was also shown to be hypersensitive to berberine (Hu et al., 2014). Such hypersensitivity is one advantage of berberine, as this compound can halt cancerous cell proliferation by blocking DNA synthesis. Berberine also induces a significant increase in double strand breaks and instability, likely because of its ability to intercalate into DNA, which can reduce the transcription level of genes such as hTERT and Rev3 (Hu et al., 2014). However, berberine may have harmful effects towards healthy cells, especially those undergoing normal division such as immune cells, which require continued DNA synthesis. Nevertheless, while berberine could fight some types of cancer, excessive berberine doses could promote development of other kinds of cancer, especially those related to blood circulation, including leukemia and lymphoma. As such, further investigation is needed to determine optimal doses that maximize the therapeutic effect while minimizing harmful side effects.

Lastly, berberine has also been shown to decrease levels of oncogenic H-Ras and c-Fos in T24 bladder cancer cells (Lin et al., 2012). c-Fos and c-Jun are subunits of AP-1 (Fig. 3), another activator of gene expression. A previous study showed that berberine can downregulate c-Fos expression (Mahata et al., 2011), which is in agreement with the finding that AP-1 expression was decreased by berberine treatment of MDA-MB-231 cells (Kim et al., 2008).

2.7. Berberine and microRNAs

MicroRNAs are small (21–23 nucleotides) non-encoding RNA molecules that are transcribed by RNA polymerase II. Generally, miRNAs bind to 3′ untranslated regions (UTRs) of messenger RNAs (mRNAs) to suppress mRNA translation (Iorio et al., 2005). miRNAs play an important role in the development and progression of various cancers, including breast cancer, by promoting continuous

cell divisions (KEGG website). microRNAs can be sub-divided into two categories: tumor suppressor miRNAs and oncogenic miRNAs. To date, a number of miRNAs have been associated with breast cancer and these, as well as their most important targets, are listed in Table 3.

miR-21 is one of the most important microRNAs and is involved in breast, colon, lung, pancreas, prostate, and stomach cancers (Liu et al., 2013a). miR-21 is a crucial factor that promotes resistance to chemotherapy in cancer cells. Previous studies showed that when miR-21 expression in breast cancer cell lines was decreased, the sensitivity of these cells to chemotherapy increased (O'Day and Lal, 2010; Corcoran et al., 2011). In addition, miR-21 levels in MCF-7 breast cancer cells were increased, which downregulated p53 expression (Iorio et al., 2005; Vimalraj et al., 2013). miR-21 can also activate key anti-apoptotic factors such as Bcl-2 (Hu et al., 2013).

In contrast, miR-145 is only expressed in normal breast cells, but not breast cancer cell lines (Iorio et al., 2005). On the other hand, miR-125b was found to have the same level of expression in both normal breast cell lines and the MDA-MB-231 breast cancer cell line, whereas breast cancer cell lines such as MCF-7 and BT-20 had downregulated miR-125b expression (Iorio et al., 2005).

Recently, studies on the effects of natural compounds on the expression of microRNAs related to several cancer types have been investigated. Pomegranate extract reduced miR-27a expression in two types of breast cancer cell lines, BT474 and MDA-MB-231 (Banerjee et al., 2012), which in turn led to downregulation of Sp proteins and Sp-regulated genes, and eventually a critical reduction in NF-κB activation that decreased cell proliferation.

The effects of berberine on microRNAs are not well understood and only a few reports on berberine and microRNAs have been published to date. The effects of berberine have been investigated only for miR-21 and miR-21-3p cancer-related microRNAs (Lo et al., 2013a; Liu et al., 2013a; Hu et al., 2013), with berberine-based inhibition of miR-21 having been reported for ovarian cancer (Liu et al., 2013a). Another study demonstrated that berberine suppressed cell proliferation and IL-6 secretion in human myeloma cells by downregulating miR-21 levels, which subsequently led to increased levels of p53 (Hu et al., 2013).

In a separate study, the effects of berberine on miR-21-3p cancer-related microRNAs in hepatoma cells have been shown wherein berberine increased the levels of miR-21-3p expression that led to growth suppression and apoptosis induction in liver cancer cells (Lo et al., 2013b). The effect of berberine on microRNAs related to breast cancer, however, awaits further detailed investigations.

2.8. Berberine metabolism, toxicity and Cytochrome P450

Despite an *in vivo* experimental trial in humans, to characterize the antineoplastic properties of berberine and its ability to suppress cyclin B1, Cdc2, and Cdc25c in colorectal cancer (Cai et al., 2014), as well as, to determine other advantages of berberine for induction of cancer cells death as discussed earlier, its effects and efficacy on liver enzymes is not clearly understood from the literature. Thus, the toxicity of berberine and its harmful effects on various tissues should be elucidated.

Cytochrome P450 (CYP) enzymes, located in smooth endoplasmic reticulum play a crucial role in drug processing and elimination of xenobiotics. Among all the enzymes belonging to CYP superfamily, Cyp2D is a major player in most drugs elimination (Fig. 5). Berberine metabolism is mostly directed by Cyp2D6 in liver cells (Guo et al., 2011a) compared with other CYPs such as Cyp2D1, Cyp3A1/2, Cyp3A4, Cyp1A2, and Cyp2C19 (Liu et al., 2009a; Guo et al., 2011a). The efficacy and toxicity of berberine derivatives on normal cells remain unknown *in vivo* and await additional evaluation. Guo et al. (2011a) reported that 11 berberine metabolites were observed in mouse urine and feces, and most of these metabolites were demethylated products (Liu et al., 2009a; Guo et al., 2011a). During oxidative demethylation and demethylenation of berberine, Liu et al. (2009a) demonstrated that subsequent glucoronidation produced conjugated metabolites. Once berberine co-administered, these conjugated products may lead to drug–drug interactions (Liu et al., 2009a; Zhou et al., 2012). Drug–drug interaction may affect berberine small intestinal absorption and subsequent elimination by the liver (Zhou et al., 2012). Applying human recombinant CYPs, Cyp2D6 and Cyp1A2 were the major berberine metabolizers (Guo et al., 2011a). These metabolites may also have some side effects in blood circulation. Therefore, the side-effects of berberine must be considered as an important limitation when administering berberine as an anticancer drug. The effect of Cyp2D6 may decrease the toxicity of berberine and further studies are needed to clarify whether berberine metabolites have side effects or are only excreted out.

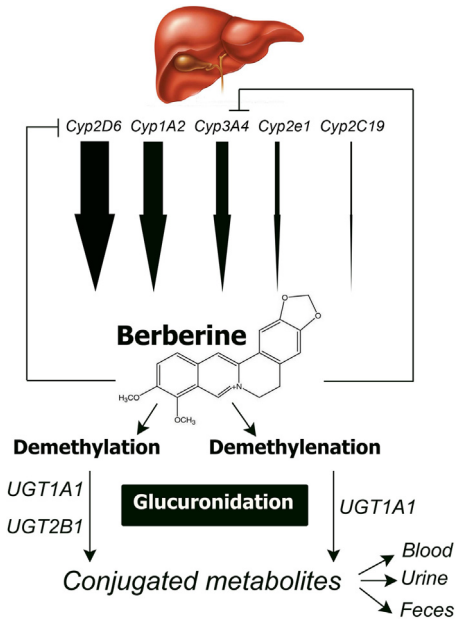

Fig. 5. Berberine metabolism. Berberine is mostly metabolized by Cyp2D6. CYP metabolizing effects on berberine are shown here by the width of arrows. Berberine can also inhibit Cyp2D6 and Cyp3A4 which can affect its metabolism with other co-administered drugs. After oxidative reaction of CYPs, subsequent glucuronidation is occurred by UGT1A1 and UGT2B1. Demethylated metabolites are conjugated by both, but demethylenated metabolites are conjugated by UGT1A1.

Other studies reported that berberine play a role in decreasing Cyp2D6 mRNA expression. After oral administration of berberine to mice, study has shown that the highest dose of berberine (300 mg/kg) affected some cytochrome P450 mRNAs. No liver damage was observed with dose up to 300 mg/kg (Guo et al., 2011b). Guo et al. (2011b) also reported that Cyp3a11 and Cyp3a25 mRNAs expressions were decreased, and there was an increase in Cyp1a2 mRNA expression at highest dose. Berberine could also decrease enzyme activities of Cyp3a11 and Cyp2d22 in mice. Cyp3a11 and Cyp2d22 are homologs of human Cyp3A4 and Cyp2D6, respectively. Guo et al., 2012 reported that berberine decreased human Cyp2D6 mRNA expression. These studies showed that berberine in a low dose may be metabolized and at a high dose may affect its metabolism by decreasing CYP activities which can affect the anticancer properties of berberine, especially when administered in combination therapy with other chemotherapeutics antagonist which might interfere with its action.

Berberine can also affect metabolism of estrogen as a major risk factor in hormone-dependent breast cancer. Estrogen is metabolized

Table 3
microRNAs involved in breast cancer. Up-regulation and/or down-regulation of certain microRNAs abnormally induces cell proliferation, gene expression, and cell signaling pathways, which can lead to cancer development. This information was collected from the KEGG database (Lee and Dutta, 2009) to show the importance of microRNAs in regulating main cell processes. However, the effects of berberine on these breast cancer-associated microRNAs still remain unclear.

MicroRNAs	Overall role	Inhibited protein	The role of inhibited protein
Up-regulated microRNAs (oncogenic microRNA)			
miR-181	Normal mammary	ATM	DNA repair
miR-155	Normal mammary	SOCS1	Suppressor of cytokine signaling 1
miR-10b	Metastasis	HOXD10	Transcription factor
miR-373	Metastasis	CD44	Cell–cell interaction
miR-520	Metastasis	CD44	Cell–cell interaction
miR-103	Metastasis	Dicer	microRNA processing
miR-107	Metastasis	Dicer	microRNA processing
miR-21	Metastasis	TPM1	Cell cytoskeleton
		PDCD4	Apoptosis induction
		SerpinB5	P53 signaling pathway
miR-31	Metastasis	Fzd3	Wnt signaling pathway
		ITGA3	PI3K/Akt signaling pathway
		RDX	Cell cytoskeleton
		RhoA	Cell cytoskeleton
miR-193b	Metastasis	uPA	NF-κB signaling pathway
miR-221	Therapy resistance	Kip1/p27	Cell cycle inhibitor
miR-222	Therapy resistance	Kip1/p27	Cell cycle inhibitor
miR-125b	Therapy resistance	BAK1	Apoptosis activator
Down-regulated microRNAs (tumor suppressor microRNA)			
miR-200	Normal mammary	Bmi-1	Regulate cell cycle inhibitors (p16/p19)
		ZEB1	Transcription factor
		ZEB2	Transcription repressor
		FOG2	Transcription factor
Let-7	Normal mammary	Ras	Signaling pathway
		HMGA2	Gene expression
miR-30e	Cancer initiation	ITGB3	PI3K/Akt signaling pathway
		UBC9	NF-κB signaling pathway
miR-200	Cancer initiation	Bmi-1	Regulate cell cycle inhibitors
		ZEB1	Transcription factor
		ZEB2	Transcription repressor
		FOG2	Transcription factor
Let-7	Cancer initiation	Ras	Signaling pathway
		HMGA2	Gene expression
miR-335	Metastasis	SOX4	Transcription factor
		TNC	PI3K/Akt signaling pathway
miR-200	Metastasis	Bmi-1	Regulate cell cycle inhibitors
		ZEB1	Transcription factor
		ZEB2	Transcription repressor
		FOG2	Transcription factor
Let-7	Metastasis	Ras	Signaling pathway
		HMGA2	Gene expression
miR-451	Therapy resistance	MDR1	ABC transporter
miR-345	Therapy resistance	MRP1	ABC transporter
miR-7	Therapy resistance	MRP1	ABC transporter

by cytochrome P450 enzymes, Cyp1A1 and Cyp1B1. Recently, berberine was reported to increase the expression level of Cyp1A1 and Cyp1B1 by 16- to 52-fold in MCF-7 breast cells (Wen et al., 2014). This increase could lead to a crucial reduction in cellular concentrations of estrogen, which in turn would reduce relevant gene expression in hormone-dependent breast cancers.

3. Conclusions and future directions

The overall purpose of this review is to emphasize that herbal-based drugs are important compounds that affect cell signaling pathways and can be used in fighting cancer. Further studies on these natural compounds will be important to open new avenues for the development of novel, natural drugs for the treatment of various cancers, especially breast cancer.

The potential application of berberine and definition of its targets in breast cancer still remains an important question. A better understanding of the gene networks and cellular pathways regulated by berberine will undoubtedly enable a better understanding of breast cancer pathogenesis and therapy. According to the literature, such complex networks exist in cells to manage cell proliferation, although the amount of information concerning the effects of berberine on these networks, particularly those in breast cancer, remains small.

Berberine can inhibit Ras/MAPK and PI3K pathways, which play a major role in promoting gene expression and may be excessively activated in cancer cells. Berberine also suppresses the activation of some cell growth factor receptors (EGF receptor, Her2/neu, and VEGF receptor) and increases tumor suppressor levels to arrest cancerous cell cycle progression (p53, Cip1/p21, Kip1/p27, and Rb). Berberine promotes apoptosis by increasing the level of apoptosis-related molecules such as caspases, Bax and Smac/DIABLO. However, Rho/Cdc42 and Wnt/β-catenin signaling pathways are crucial signaling pathways that regulate cell mortality, and induction of these pathways leads to metastasis. Berberine can suppress activation of Wnt/β-catenin signaling pathway while increasing the amount of E-cadherin, a cell membrane adhesion protein (Qi et al., 2014). The effects of berberine on cell cytoskeleton signaling pathways related to integrin and actin polymerization have not been extensively studied, and information on the effects of berberine on these proteins may help decipher its potential

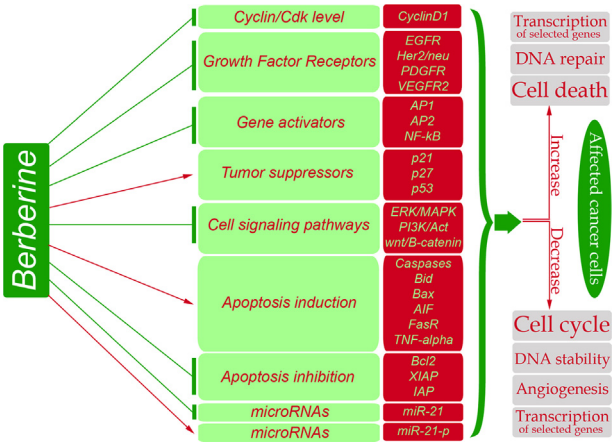

Fig. 6. An overall summary of the molecular effects of berberine and anti-cancer outcomes.

capacity as an anti-metastatic compound. Fig. 6 summarizes important genes and proteins that are affected by berberine.

In addition to metastasis, angiogenic activity is another problem posed by cancer cells. Vascular endothelial growth factor A (VEGF-A) and fibroblast growth factor 2 (FGF-2) are known to be synergistically involved in angiogenesis (Kano et al., 2005; Jackson and Sugrue, 2006). In leukemia, berberine can decrease VEGF receptor levels (Lin et al., 2012), although the effect of berberine on FGF receptors awaits exploration. By studying the effects of berberine on such growth factors, we may be able to suppress the growth of cancer cells more effectively. The effect of berberine on factors involved in the methylation of CpG islands located at gene promoters, and its effect on epigenetic changes, is another important question that requires further attention.

As discussed earlier, microRNAs act as regulators of cell signaling and post-transcriptional modifications, and a better understanding of their roles could yield novel approaches for treating cancer. microRNAs appear to be optimal drug targets and could be used in combination with other drugs or therapies to reduce the treatment time period. The effects of berberine on microRNAs associated with breast cancer have not been studied yet, and assessment of microRNA–berberine interactions would be a crucial research topic for future investigations. Furthermore, berberine may affect the level of two categories of microRNAs in breast cancer, oncogenic and tumor suppressor microRNAs, although in general the effects of berberine on microRNAs related to most cancers have not been extensively explored.

A main concern of anti-cancer drugs is their harmful effects on normal tissues. To address this problem, purposeful drug delivery should enable the targeting of certain cell types by nanoparticles and liposomes. Several studies have explored the delivery of natural compounds such as berberine and curcumin by drug-containing vesicles (Ma et al., 2013; Naksuriya et al., 2014). To design such delivery systems, drug targets should be extensively characterized. ABC transporters (ATP-binding cassette transporters) are a family of membranous proteins that actively efflux drugs that enter cells and are major factors in drug resistance. Ma et al. (2013) designed berberine-containing liposomes that can directly target mitochondrial proteins. By understanding the targets of berberine and the mechanisms by which it could be used to control breast cancer, the effectiveness of berberine in breast cancer treatment can be determined. Short-term clinical treatment methods using berberine at doses that produce few serious side effects could be a promising therapeutic strategy, although further study is needed to determine mechanisms by which berberine acts, as well as optimal dosages and delivery methods.

Determination of the half inhibitory concentration, denoted by IC_{50}, can inform decisions about the drug concentrations that can efficiently kill 50% of cells. Different IC_{50} values for the same cell line can be obtained, likely due to different cell conditions, while different IC_{50}s for berberine have also been reported for different cell lines (MCF-7, IC_{50}=20 μmol/l, 72 h; MDA-MB231, IC_{50}=20 μmol/l, 48 h; HeLa, IC_{50}=7.2 μmol/l, 48 h) (Sun et al., 2009). Targets of berberine and their mechanisms to control breast cancer, and the inhibitory effects of berberine in both healthy cells and cancerous cells should be determined. Therefore, additional study is needed to design short-term treatment methods using berberine that do not have serious side-effects.

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