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Honokiol, a multifunctional tumor cell death inducer

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Honokiol is a small-molecule pharmacologically active component which has various medicinal applications. Increasing interest is paid on its multifunctional anti-tumor effects including inducing tumor cell death, anti-angiogenesis, anti-migration and anti-multiple drug resistance. We addressed a brief summary of the anti-tumor actions and potential applications of honokiol. This review is mainly focused on the multiple types of cell death induced by honokiol, and its potential role in overcoming multiple drug resistance.

1. Introduction

Honokiol (HNK) is a pharmacologically active small molecule isolated from the Chinese traditional medicinal herb-Houpu. It has been reported as an agent with multiple medicinal applications including anti-inflammatory, anti-anxiety and anti-tumor effects, in preclinical studies. No perceivable toxicity has been detected when it was used in therapeutic doses. Taking its anti-tumor effect into consideration, mechanisms such as tumor-cell death induction, anti-angiogenesis, anti-migration (Singh and Katiyar 2011) and anti-multiple drug resistance are all taking participation. Among all the anti-tumor effects, HNK-induced cell death is the most fundamental one. The principal aim of this review is to summarize the basic anti-tumor functions of HNK, especially the association with tumor-cell death and multiple drug resistance.

2. Honokiol induced cell death

Cell death is always classified by morphological, enzymological, functional and immunological characteristics (Melino 2001). According to the recommendations of the Nomenclature Committee on Cell Death, different types of cell death include apoptosis, autophagy, cornification, necrosis and other types (Kroemer et al. 2009). Accumulating evidence indicates cell death induced by chemotherapy or radiotherapy is most related to anti-cancer therapy. HNK plays an important role as a multifunctional tumor cell death inducer within its numerous cytotoxicity and anti-tumor effects. After the first report of its anti-tumor effect in 1994 (Hirano et al. 1994), more attention has been paid on this drug. Recent studies mostly focused on its apoptosis-induction effect. Honokiol has been reported to exhibit a competent cytotoxicity by inducing cell apoptosis in a variety of human tumor cell lines *in vitro* or *in vivo*, such as gastric cancer, breast cancer, hepatoma.

2.1. Honokiol and apoptosis

The most frequently reported type of tumor cell death induced by HNK is apoptosis, through death receptor pathway and mitochondrial pathway (Fig. 1), which contains caspsase-dependent and independent pathways. Apoptosis is characterized by a series of distinctive morphological changes including condensation of cytoplasmic and chromatin, nuclear fragmentation, membrane blebbing, and formation of apoptotic bodies. Most studies of HNK-induced tumor cell death demonstrated the typical morphological features like apoptotic bodies (Hibasami et al. 1998) and karyopyknosis (Chen et al. 2011), which confirmed apoptosis. Characteristic DNA fragmentation was also detected in HNK-induced tumor cell death by DNA bledding (Chen et al. 2011; Hibasami et al. 1998). Both death receptor pathway and mitochondrial pathway stimulate the activation of the cysteine protease family of caspases (Hirano et al. 1994). Activation of pro-apoptotic "death receptors" (such as Fas, TNF receptor associated factor (TNFR) 1 and TRAIL-R1/-R2) at cell surface results in activation of caspase-8 or 10, and in turn induces the activation of caspase-3 and 7 (Iannolo et al. 2008). Caspase-8 is often activated in the HNK-induced extrinsic pathway (Shigemura et al. 2007; Battle et al. 2005; Park et al. 2009). TRAF is an important component in TNFR1 inducing apoptosis signal pathway. TRAF activates NF-κB, which suppresses TNF induced apoptosis. HNK promoted apoptosis which was induced by TNF (Ahn et al. 2006). Ahn and coworkers found HNK suppressed NF-κB activation and down-regulated the NF-κB regulated gene expression, such as those associated with anti-apoptosis (Bcl-xL, Bcl-2, c-FLIP and TRAF1), proliferation, invasion and angiogenesis. Raja et al. (2008) demonstrated that HNK played as a sensitizer in death receptor-mediated apoptosis of tumor cells. Death-inducing signaling complex (DISC) is an essential component in the extrinsic apoptosis pathway. Protein cellular FLICE-inhibitory protein (c-FLIP) is structurally similar to caspase-8 but enzymatically inactive. It negatively modulates the functions of DISC and suppresses death receptor induced apoptosis. They found when combined with TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), Fas ligand or an agonistic anti-Fas antibody, HNK enhanced their apoptosis-induced activities. HNK further down-regulated c-FLIP through proteasome-mediated degradation or increased ubiquitination. Enhancing the expression of c-FLIP inhibited

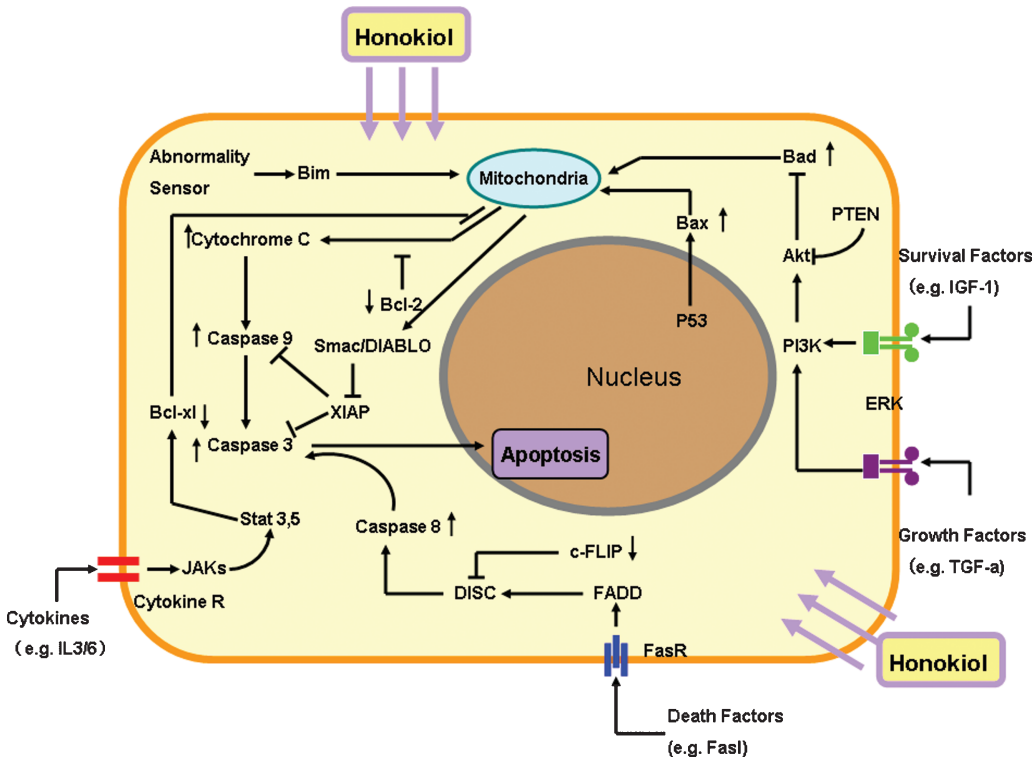

Fig. 1: Honokiol-induced tumor cell apoptosis: '↑' refers to 'up-regulated' and '↓' refers to 'down-regulated'

the "sensitizer" effect of HNK in TRAIL-associated apoptosis. They thus presumed the down-regulation of c-FLIP was a key procedure in the process of HNK-modulated death receptor-mediated apoptosis (Raja et al. 2008). The intrinsic pathway is characterized by mitochondrial dysfunction (such as mitochondrial outer membrane permeabilization, MOMP) which opens the mitochondrial permeability transition (MPT) pore and allows the release of two groups of pro-apoptotic proteins from the inter-mitochondrial membrane space. The first group of proteins contains cytochrome c, Smac/DIABLO, and serine protease HtrA2/Omi. These proteins activate the caspase-dependent mitochondrial pathway. Binding of cytochrome c with apoptotic protease-activating factor-1 (APAF-1) results in the formation of apoptosomes complex and activates downstream caspases rapidly. That, in turn, triggers the degradation of cellular structures. The Bcl-2 protein family regulates the activation of the intrinsic pathway of apoptosis by either promoting or preventing (Danial and Korsmeyr 2004) the release of mitochondrial proteins. After released from the mitochondrion, Smac/DIABLO binds to IAPs (inhibitor of apoptosis proteins) and suppresses their effects, which blocks apoptotic signaling by inhibiting caspases (van Loo et al. 2002). Mitochondrial dysfunction and endoplasmic reticulum stress participate in the HNK-induced intrinsic pathway of apoptosis (Chen et al. 2010). Anti-apoptotic members of the Bcl-2 protein family, such as Bcl-2/Bcl-XL are down-regulated (Chen et al. 2010; Park et al. 2009; Yang et al. 2002), while pro-apoptotic members (Bax, Bak, Bad and tBid) are up-regulated (Yang et al. 2002; Chen et al. 2010; Park et al. 2009; Li et al. 2008). Release of cytochrome c is identified in HNK-induced apoptosis (Yang et al. 2002; Mannal PW 2011), and caspase-3 and 9 are often activated in this process(Mannal PW 2011; Yang et al. 2002; Li et al. 2008). Mannal et al. (2011) demonstrated the anti-tumor effect of HNK though apoptosis in melanoma cell lines (SK-MEL2 and MeWo). They detected a significant increase in caspase activity after exposure to HNK. Mitochondrial depolarization and increased cytochrome c were key events in HNK induced death. Chen et al. (2010) reported that HNK had anti-tumor effects and induced apoptosis in human chondrosarcoma cell lines. The expressions of Bax and Bak were up-regulated after exposure to HNK, while Bcl-XL was down-regulated. Moreover, they demonstrated that mitochondrial dysfunction and endoplasmic reticulum stress were key events in HNK-induced apoptosis. Bid exerts a bridge-like effect which connects these two pathways. Evidence has indicated that there is a cross-talk between the two and they share some molecules important in these two pathways (Twiddy et al. 2004). Some authors demonstrated both the death receptor pathway and mitochondrial pathway were involved in the HNK-induced apoptosis (Park et al. 2009; Shigemura et al. 2007). Shigemura et al. (2007) demonstrated that HNK could induce caspase-dependent apoptosis through both extrinsic and intrinsic pathways, which was confirmed by activation of caspase-3, 8, 9 and cleavage of PARP (poly-adenosine diphosphate ribose polymerase) in prostate cancer cell lines. Caspsase-independent apoptosis is also involved in HNK-induced cell death. Pro-apoptotic proteins of the second group released from mitochondrion, such as AIF and endonuclease G, are important effectors in the caspsase-independent apoptosis. Both proteins translocate to the nucleus and response to the formation of DNA fragmentations. They function in a caspsase-independent manner. HNK induced caspase-independent apoptosis was first reported by Ishitsuka et al. (2005). They found both caspase-dependent and independent apoptosis took part in the process of HNK-induced cytotoxicity in human multiple myeloma cell lines (including chemo-resistant cell lines and primary cultured cells). Pan-caspase inhibitor (z-VAD-fmk) could not fully suppress HNK-induced apoptosis. Release of proapoptotic protein apoptosis-inducing factor (AIF) from mitochondrial to cytoplasm was detected by Western blotting. What encouraged us mostly was that HNK had no cytotoxicity on normal PBMCs in therapeutic doses (exposure to HNK in concentrations of 20 μg/mL or 40 μg/mL for 48 h).

Other signal transduction pathways are also activated in the HNK induced apoptosis. A study about the anti-tumor activity against human breast cancer cell lines was carried out by Wolf et al. (2007). Proliferation inhibition was detected in all

the breast cancer lines tested. The IC50 of HNK to HR-negative, p53-mutated cell lines (MDAMB-231, SK-BR-3) were lower than the others. Both caspase-dependent and independent apoptosis were involved. To find the functional actions of HNK, the expression of EGFR, total and phosphorylated ERK2, and phosphorylated AKT, after MDA-MB-231 cells treated with different concentrations of HNK, were analyzed by Western blotting. The expressions of the first two were down-regulated. Hence, they suggested that inhibition of the expression of EGFR and its downstream pathway, the MAPK cascade, was a key event in the HNK-induced apoptosis process. HNK showed anti-tumor effects on different human breast cancer cell lines, not only normal cell lines, but also drug-resistant cell lines (such as adriamycin-resistant and tamoxifen-resistant cell lines) (Liu et al. 2008). Researchers found that the effect of HNK induced apoptosis was associated with the expression level of HER-2. Down-regulated HER-2 expression by siRNA or combination with EGFR/HER-2 kinase inhibitor lapatinib enhanced HNK induced apoptosis in HER-2 over-expressed BT-474 cells. The attenuation of PI3K/Akt/mTOR signaling transduction pathway through down-regulated expression of phosphorylated Akt and up-regulated expression of PTEN was one of the potential mechanisms associated with the cytotoxic effects of HNK. Aberrant activation of PI3K/Akt/mTOR pathway is associated with the development of resistance to therapeutics such as trastuzumab and tamoxifen. HNK might be a potential agent to overcome trastuzumab or tamoxifen resistance. Park et al. (2009) analyzed the signal transduction pathway which was involved in HNK induced cell cycle arrest and apoptosis in human breast cancer cell line MDA-MB-231. They found that after exposure to HNK, the expressions of phosphorylated c-Src, EGFR and Akt were down-regulated, which led to the inhibition of downstream signal transduction. Similar to others, they found expressions of caspase-3, -8, -9, Bid and Bcl-2 changed during HNK induced apoptosis. In the study of Deng et al. (2008), the p38 mitogen-activated protein kinase pathway was involved in the process of HNK induced human hepatoma cell line (hepG2) apoptosis.

2.2. HNK and other kinds of cell death

Besides apoptosis, HNK also induces other kinds of cell death in tumor cells. That initiates a new understanding of its anti-tumor mechanism.

Necrosis (necrotic cell death) was once defined as an uncontrolled and accidental form of cell death. Accumulating evidence suggests that necrosis is regulated by a couple of signal transduction pathways (Festjens et al. 2006; Golstein and Kroemer 2007). Thus, some authors introduce a new term "necroptosis" to describe programmed necrosis (Vandenabeele et al. 2010). The characterized morphological features of necroptosis include increased cell volume due to swelling of cytoplasimic organelles, plasma membrane rupture, dilatation of mitochondria and endoplasmic reticulum. The induction and regulation mechanism of necroptosis remains poorly understood. Some phenomena are implicated in necroptosis including change of mitochondrial membrane permeabilization, alteration of lysosomes and nuclear, upregulaion of calcium concentration in cytoplasm (Festjens et al. 2006; Golstein and Kroemer 2007). Necroptosis is difficult to be defined due to the lack of characteristic biochemical features. But it is characterized by distinctive morphological features, which show early plasma membrane permeabilization. Previous reports of our group showed that HNK induced necrotic cell death through the mitochondrial permeability transition pore in HL60, MCF-7 and HEK293 cell lines (Li et al. 2007). We demonstrated the rapid loss of integrity of plasma membrane was the characterized morphological feature in HNK-induced death. The cytological change of HNK-induced necrosis was characterized by a rapid loss of mitochondrial membrane potential, which was mechanistically modulated by the mitochondrial permeability transition pore (MPT pore). Cyclophilin D (CypD) was the critical modulator, which regulated the MPT pore in HNK-induced necrosis procedure. We provided evidence that cyclosporin A (inhibitor of CypD) and RNA interference of CypD effectively prevented the loss of mitochondrial membrane potential and blocked HNK-induced cell death.

Originally, the introduction of the conception "paraptosis" is used to describe a particular form of programmed cell death. The morphological and biochemical manifestations of paraptosis are distinct from typical apoptosis (Sperandio et al. 2000). Extensive cytoplasmic vacuolization and mitochondrial swelling are major morphological changes of paraptosis, but other morphological features of apoptosis (such as apoptotic bodies) are always absent (Sperandio et al. 2000). Moreover, the proceeding of paraptosis cannot be blocked by the caspase inhibitors or up-regulated expression of antiapoptotic Bcl-2-like proteins (Sperandio et al. 2000, 2004). Frequently, the expression of IGFR-1 is apparently a key event in triggering of paraptosis process. Specific members of the MAPK family (Sperandio et al. 2004) are probably involved in the signaling process. Wang et al. (2010) demonstrated that lower concentrations of HNK induced paraptosis, whearas both paraptosis and apoptosis were induced at a higher concentrations in leukemia cell lines. After exposition to lower concentrations, cells experienced death processes with distinctive morphological alterations, such as cytoplasmic vacuolization and endoplasmic reticulum swelling. Both types of cell death induced by HNK were associated with membrane-associated cytotoxicity. The authors presumed that 'The two death processes may happen in sequence at lower concentrations and in parallel with the increase of HNK concentration' (Wang et al. 2010), and confirmed the existence of a "cross-talk" between HNK induced-apoptotic and non-apoptotic programmed cell death in leukemia cells.

3. HNK and multiple drug resistance in cancer

The development of multiple drug resistance (MDR) is still a major challenge in cancer therapy. MDR in cancer therapy is defined as 'simultaneous resistance to several structurally unrelated drugs that do not have a common mechanism of action' (Gottesman et al. 2002). Numerous mechanisms are involved in the development of MDR including drug transporter-mediated decreased uptake and increased efflux of anti-tumor drugs, activation of detoxifying systems and DNA repair mechanisms, drug-induced apoptosis deficiency (Gottesman et al. 2002) and others. Most of the anti-tumor agents are inducers and substrates of drug transporters or ultimately induce a dominant apoptosis (Hu et al. 2008; Hu and Xuan 2008). Drug-transporters and drug-induced apoptosis deficiency have been studied and reviewed most completely (Igney and Krammer 2002; Brown and Attardi 2005; Szakács et al. 2006; Beguleya 2010). Drug transporters either catalyze drug efflux from the cell or encapsulate and eliminate the drugs (Fig. 2 A). After the first identification of the membrane transporter P-glycoprotein, at least 48 structurally related transporters belonging to ATP-binding cassette (ABC) family have been identified. There are three subfamilies relevant to multiple drug resistance (Beguleya 2010). The over-expression of drug transporters on tumor cells dilutes a range of structurally unrelated anti-tumor drugs. Numerous drugs are designed to overcome transport-mediated MDR, which are often subdivided into three generations (such as dexverapamil, valspodar or biricodar belonging to the second generation, tariquidar,

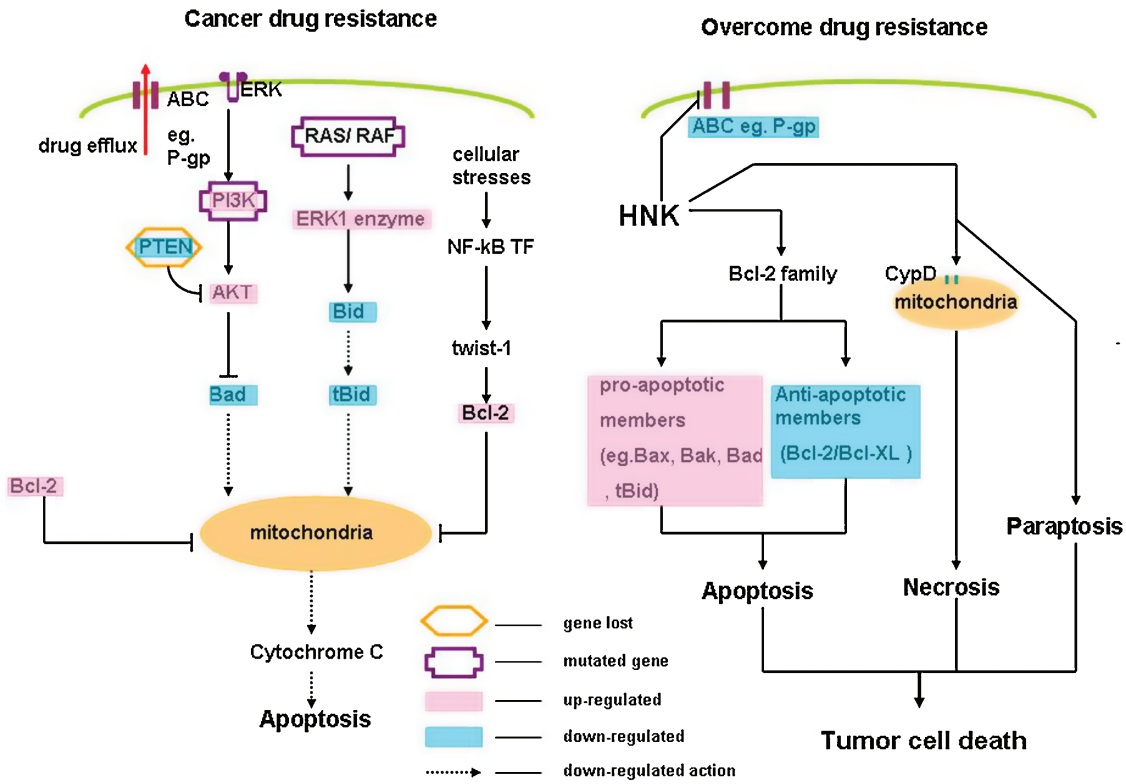

Fig. 2: HNK and Multiple Drug Resistance (A: mechanisms of drug-transporters and drug-induced apoptosis deficiency in drug- resistant tumor cells; B: the potential role of HNK in overcoming MDR)

zosuquidar or laniquidar belonging to the third generation). Most of them failed to show clinical efficacy in a couple of Phase II trails (Gandhi et al. 2007; Ruff et al. 2009). The study of our group (Xu et al. 2006) showed that HNK down-regulated the expression of P-glycoprotein at both mRNA and protein levels in MDR breast cancer cell line (Fig. 2B). Honokiol simply down-regulated the expression of P-gps. It was different from the dual functions of inhibiting P-gp's drug-pump and expression of verapamil or curcuminoids. And the intracellular drug accumulation and sensitivity to adriamycin were partially restored due to the HNK-induced down-regulation of P-gp expression.

Besides drug-transporters, cell death deficiency (especially apoptosis deficiency) is probably another key event in chemotherapy resistance (Fig. 2A). Cellular death is the substantial pharmacological purpose of chemotherapy. Once acquired apoptotic resistance, cancer cells are able to block the downstream signal transduction events induced by chemotherapy drugs and escape from the drug-induced apoptosis. Generally, alterations of two major signal transduction pathways, induction of "stress" signaling pathways associated with p38 kinase (Sharma et al. 2006; Olson et al. 2004) or suppression of "survival" signaling pathways coordinated by PI3K and extracellular-regulated kinase-1 (ERK1) (Hersey et al. 2006), are involved in the proceeding of cytotoxic effects of anti-tumor drugs. Inhibition or promotion of activity of the Bcl-2 proteins family and related molecules can be balanced through the modulation of these pathways mentioned above (Danial 2007). The proteins of the Bcl-2 family and related molecules are involved in regulation of the permeability of mitochondrial membranes in the apoptosis pathway. Cytotoxic anti-tumor drug functions through disrupting the mitochondrial permeability transition pore (MPT pore) by modulating these two signal pathway (Forte and Bernardi 2006). As a result, proteins related with the apoptosis process (such as cytochrome C, endonuclease G, and apoptosis-inducing factor (AIF)) will be released into the cytoplasm and induce cell death through various pathways. The efficacy of anti-tumor drugs used to induce the cell death cascade may be compromised by the change of signal pathways by any tumor cell itself. Increased activity of AKT (PKB) and phosphorylation of Bad can be induced by gene mutation for phosphoinositide 3-kinase (PI3K) or gene deletion for PTEN, a phosphatase that regulates the activity of PI3K. Reduction of unphosphorylated Bad results in the decrease of mitochondria permeability transition and thus increases the death resistance of tumor cells. Likewise, activation of the ERK1 enzyme and inactivation of phosphorylated Bid can be caused by activating mutations in the RAS or RAF genes, which protects mitochondria from the permeability transition. Also, phosphorylation of Bcl-2 and induced over-expression of Bcl-2 itself provide a mechanism of resistance from cell death (Pham et al. 2007; Osford et al. 2004). HNK also induces drug-resistant tumor cells to apoptosis, which is thought to show the ability to overcome apoptosis deficiency (Fig. 2B). Ishitsuka et al. (2005) found that after exposed to HNK, apoptosis was induced in the SU-DHL4 cell line (a cell line of human multiple myeloma which has low levels of caspase-3 and-8 and drug resistance). Liu et al. (2008) demonstrated that HNK showed anti-tumor effect which associated with induction of caspase-dependent apoptosis in different human breast cancer cell lines including drug-resistant cell lines (such as adriamycin-resistant and tamoxifen-resistant cell lines).

As 'cancer drug resistance is a complex, dynamic, and elusive system' (Hu and Xuan 2008), it is difficult to restore the efficacy of chemotherapy simply by reactivating apoptosis or inhibiting drug transporters. Since there are several cell death pathways with totally different molecular mechanisms, it is possible that cancer cells resistant to one kind of cell death may be susceptible to others. This hypothesis has been proved by a small molecule necroptosis inducer, Shikonin (Hu and Xuan 2008; Hu et al. 2007). HNK, as our group (Li et al. 2007) and Wang et al. (2010) demonstrated, also induce some kinds of nonapaptotic cell death in cancer cells such as necrosis or paraptosis. So it is a potential novel drug which simultaneously activates multiple death path-

ways and overcomes MDR when combined with conventional chemotherapy drugs (Fig. 2B).

4. Conclusions

Honokiol is a pharmacologically active small molecule with multifunctional anti-tumor effects. It could promote not only apoptosis, but also necrosis and paraptosis of tumor cells. Therefore, HNK can be considered as a potential novel drug which simultaneously activates multiple death pathways and overcomes MDR. In the future, more attention will be paid on its synergistic sensitizing effects with other chemotherapeutic drugs in preclinical study. Research on the toxicological effect of Honokiol is also required to investigate the security and maximum tolerated dose of Honokiol.

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