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Review

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Flavonoids as potential anti-hepatocellular carcinoma agents: Recent approaches using HepG2 cell line

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ABSTRACT: Numerous studies have documented that in cancer therapy flavonoids extracted from traditional Chinese medicine have anti-tumor activity or can enhance efficiency of chemotherapy in combination with chemotherapeutics. Thus, an awareness of flavonoids is needed by physicians and medical researchers. This review provides evidence about anti-hepatocellular carcinoma activity of flavonoids. First, as a common employed *in vitro* model, profile of HepG2 is shown. Second, the intracellular signaling pathways induced by flavonoids which inhibit the HepG2 cell line are summarized. Third, study situation of anti-HBV/HCV activity of flavonoids is shown. Our review is aimed at providing an understanding of anti-HBV/HCV activity and anti-HCC mechanisms of flavonoids, and an outlook on flavonoids application on cancer therapy.

Keywords: Flavonoids, herb medicine, hepatocellular carcinoma, HepG2

1. Introduction

Hepatocellular carcinoma (HCC) is one of the major health threats worldwide, especially in East Asia. Although chemotherapy is one of major conventional HCC therapies, the strong side effects and the emergence of drug resistance are serious problems. Meanwhile, hepatitis B virus (HBV) infection accounts for about 60% of the total liver cancer in developing countries and for about 23% of cancer in developed countries and the corresponding percentages for hepatitis C virus (HCV) infection are 33% in developing countries and 20% in developed countries (*1*). Therefore, in the development of anti-HCC agents, the anti-HBV and anti-HCV activities as well as the low side effects should be considered.

Over thousands of years, traditional Chinese medicine (TCM) and other herbal medicines have been used to treat cancer in China, Japan, and other Asian countries. They are still widely adopted because of the advantages of high efficiency, weak side effects, easy availability, and improvement of quality of life. Recently, in Europe and USA, herbal medicines are widely accepted as a form of complementary and alternative medicine (CAM) (*2*,*3*). However, on the other hand, some disadvantages of herbal medicines left several barriers for their clinical utility, such as uncertain effective constituents and unstable efficiency.

Recently, more and more effective components from herbal medicines have been identified and one of the most interesting chemicals is a flavonoid family. Flavonoids are a group of plant secondary metabolites with variable phenolic structures and are found in fruits, vegetables, roots, stems, flowers, wine, and tea (*4*). Flavonoids are usually divided into seven classes including flavonols, flavones, flavanones, flavononol, flavanols, isoflavones, and anthocyanidins (*5*) (Figure 1). Until now, over 5,000 naturally occurring flavonoids have been extracted from various herbal medicines and their chemical structures have been confirmed. Some of these flavonoids have been reported to have activities on treatment of various diseases such as heart disease, cancer, and virus infection (*6*) as well as potential protective activity against artificially induced-liver damage (*7*,*8*). In recent years, natural products have been increasingly recognized as new remedies for enhancing the efficacy and alleviating the adverse effects of tumor therapies (*9*). Accordingly, anti-HCC effects of flavonoids have been accumulated from *in vitro* and *in vivo* research evidences. This review overlooks the recent advances of research and development on flavonoids as anti-HCC agents.

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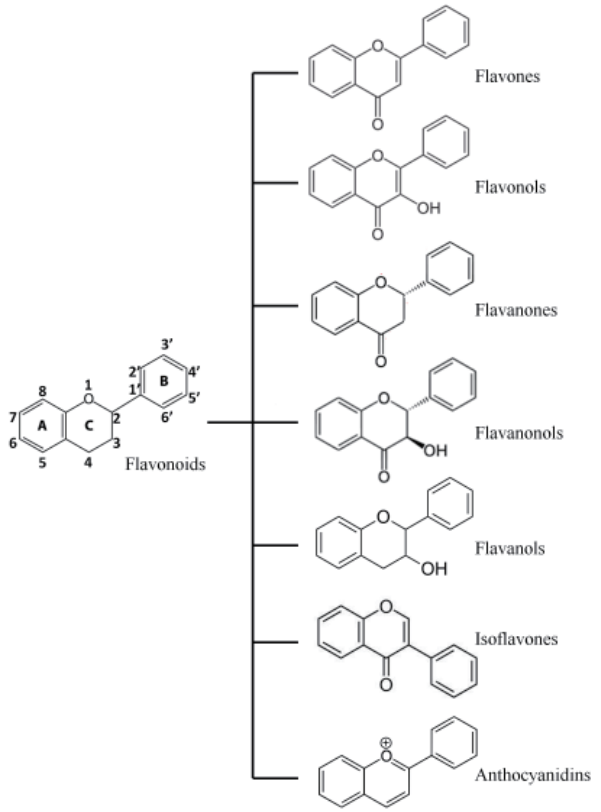

Figure 1. Molecular backbone structures of flavonoids and its subfamilies

2. HepG2 cell line, a model for investigation of flavonoids action

Since ancient times, it had been known that some TCM and other herbs could inhibit tumors, but the mechanisms were left in the dark for many centuries. Up to recent decades, molecular biological and cellular biological research gradually shed light on the mechanisms of cancer inhibition by medicines extracted from herbs. Especially very recent several years, more mechanisms of flavonoids action on HCC cell lines were illuminated and that gave a guide for selection of medicines and therapeutic methods. Among various HCC cell lines, HepG2 (ATCC No. HB-8065) is the one which has been employed most extensively in many experiments, since the cells persist a large part of cellular functions similar to those of normal hepatocytes such as expression of hepatocyte-specific cell surface receptors and synthesis and secretion of plasma proteins (*10*,*11*). Furethermore, because of the high degree of morphological and functional differentiation *in vitro*, HepG2 cell line is a suitable model to study intracellular trafficking, hepatocarcinogenesis, and drug targeting *in vitro* (*12*,*13*).

3. Signaling pathways targeted by flavonoids in HepG2 cell line

Until now, various flavonoids have been known to induce apoptosis and/or inhibit HCC cell proliferation (*14-19*). For example, flavones such as baicalein (*14*), casticin (*15*), apigenin (*16*), isoflavones such as tectorigenin (*17*), and flavonols such as galangin (*18*) and quercetin (*19*) have been reported the induction potency of apoptosis on various HCC cell lines. Various investigations using HepG2 cells have showed effects of flavonoids on signal pathways involving in apoptosis and cell proliferation. Typical mechanisms of flavonoids action on the signal pathways in HCC cells are reviewed below and the corresponding signal pathways and flavonoids are mapped in Figure 2 and summarized in Table 1, respectively.

3.1. *Unfolded protein response (UPR) pathway*

UPR pathway has been extensively implicated in proliferation, angiogenesis, and multidrug resistance of tumors (*20*). Oroxylin A, which is one of the major flavonoids produced by *Scutellaria baicalensis* Georgi (*21*), was demonstrated to depress the viability of HepG2 cells but not the normal hepatic cell line L02 (*22*). In HepG2 cells, oroxylin A treatment induced the emergence of intracellular H_2O_2 by transforming endogenous reactive oxygen species into H_2O_2, which triggered the subsequent activation of PERK-eIF2α-ATF4-CHOP branch of UPR pathway but not in normal L02 cells (*22*). PERK-eIF2α-ATF4-CHOP branch, which is a cellular stress-induced apoptosis pathway in endoplasmic reticulum (ER), includes a serial of molecules such as pancreatic ER kinase (PKR)-like ER kinase (PERK), eukaryotic initiation factor 2α (eIF2α), activating transcription factor 4 (ATF4), and CCAAT/enhancer binding protein homologous protein (CHOP). Then CHOP caused the activation of tribbles homolog 3 (TRB3) and the sequent decrease of p-Akt1/2/3 (Ser473) which is an activated form of Akt protein. Akt, an oncoprotein, is known to be frequently activated in tumor cells and positively related to poor prognosis of HCC (*23*). Since the inactivity of Akt by oroxylin A could stop boosting cancer progress and since the compound could target cancers, oroxylin A is expected as a candidate for HCC therapy (*24*). It is not a unique instance, wogonin, another *O*-methylated flavone also found in *S. baicalensis* Georgi (*25*), can also initiate UPR pathway to inhibit HepG2 cells (*26*). It is reported that wogonin touched off UPR pathway which in the next step blocked Akt phosphorylation (*27*). Overall, oroxylin A and wogonin can inhibit HepG2 cells proliferation through UPR pathway.

3.2. *Mitochondrial- and jun N-terminal kinases (JNK)-mediated apoptosis pathways*

Mitochondrial pathway of apoptosis begins with the permeabilization of the mitochondrial outer membrane (*27*). The permeabilization results in release

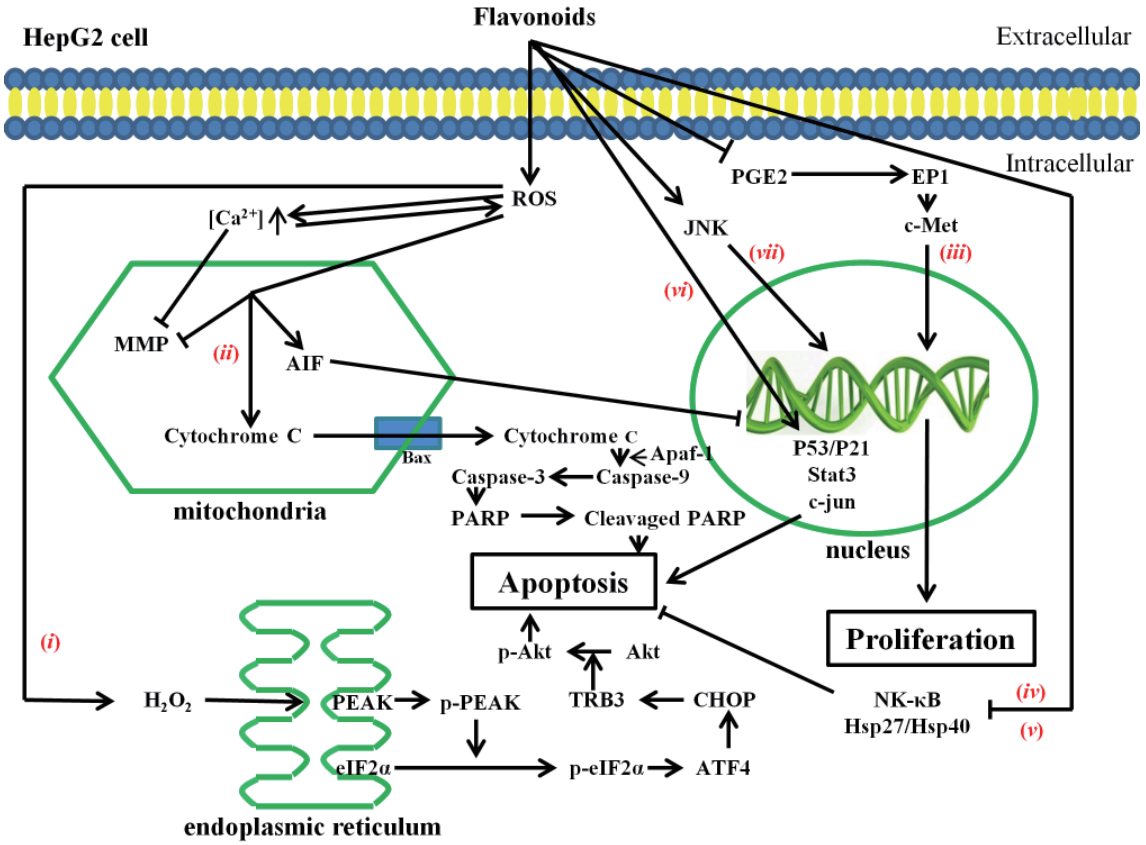

Figure 2. Signaling pathways affected by flavonoids in HepG2. Seven signaling pathways involving apoptosis and cell proliferation are mapped: (*i*) unfolded protein response pathway, (*ii*) mitochondrial-mediated apoptosis pathway, (*iii*) EGFR/c-Met signaling pathway, (*iv*) NF-κB-related pathway, (*v*) Heat shock protein-related pathway, (*vi*) tumor suppressor-related pathway, and (*vii*) JNK-mediated pathway. These pathways are reported to be affected by flavonoids and to involve in anti-HCC actions of flavonoids in HepG2 cell line. ROS, reactive oxygen species; PEAK, pancreatic ER kinase (PKR)-like ER kinase; eIF2α, eukaryotic initiation factor 2α; ATF4, activating transcription factor 4; CHOP, CCAAT/enhancer binding protein homologous protein; TRB3, chop and tribble 3; MMP, matrix metalloproteinases; AIF, apoptosis inducing factor; PARP, poly-ADP-ribose-polymerase; PGE2, prostaglandin E2; EP1, prostaglandin E2 receptor; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Hsp, heat shock protein.

Table1. Flavonoids discussed in this article

Subfamily	Flavonoids (Synonyms)	Typical origin	*Reference*
Flavone	Baicalein (5,6,7-Trihydroxyflavone)	*Scutellaria baicalensis* roots	*14*
	Casticin (3',5-Dihydroxy-3,4',6,7-tetramethoxyflavone)	*Vitex agnus-castus* leaves	*15,75*
	Apigenin (5,7,4'-trihydroxyflavone)	Orange, tea, chamomile, onion	*16*
	Oroxylin A (5,7-Dihydroxy-6-methoxyflavone)	*Scutellaria baicalensis* Georgi	*21,22,24*
	Wogonin (5,7-Dihydroxy-8-methoxyflavone)	*Scutellaria baicalensis* Georgi	*21,25,72*
	Diosmetin (5,7-Dihydroxy-2-(3-hydroxy-4-methoxyphenyl)chromen-4-one)	*Rosa agrestis* Savi	*68*
	Luteolin (2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-chromenone)	Artichoke (*Cynara scolymus*)	*68*
	5-Methoxy-(3,4"-dihydro-3",4"-diacetoxy)-2",2'-dimethylpyrano-(7,8:5",6")-flavone	*Solanum erianthum* D. Don	*71*
	Catechin (*trans*-(+)-3,3',4',5,7-Flavanpentol)	San-Huang-Xie-Xin -Tang	*73*
	Ladanein (5,6-Dihydroxy-7-methoxy-2-(4-methoxyphenyl)chromen-4-one)	*Marrubium peregrinum* L (Lamiaceae)	*74*
Isoflavone	Tectorigenin (*O*-Methylated isoflavone)	Leopard lily (*Belamcanda chinensis*)	*17,33*
	Genistein (4',5,7-Trihydroxyisoflavone)	*Genista tinctoria*	*35*
Flavonol	Galangin (3,5,7-Trihydroxyflavone)	*Alpinia officinarum*	*18*
	Quercetin (3,5,7,3',4'-Pentahydroxyflavone)	Fruits, vegetables, leaves and grains	*19,54,55*
	(−)-Epi-gallocatechin-3-gallate	Green tea	*47,48*
Flavanone	Xanthomol (1,2-Dihydropyrazolo[3,4-d]pyrimidin-4-one)	Hops	*53*
	8-Bromo-7-methoxychry (2-Bromo-α-ergocryptine)	*Oroxylum indicum* (L.)Vent.	*35*
Flavonolignans	Silymarin ((2*R*,3*R*)-3,5,7-trihydroxy-2-[(2*R*,3*R*)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydrobenzo[b][1,4]dioxin-6-yl]chroman-4-one)	*Scutellaria baicalensis* seeds	*67*

of apoptogenic proteins such as cytochrome *c* (*28*), apoptosis inducing factor (AIF) (*29*), and endonuclease G (*30*). In cytoplasm, cytochrome *c* initiates to activate various caspases such as caspases-2, -8, -9, and -10 (*31*) and the caspases in the next step cause cell death by cleaving a number of cellular proteins including DNA repair enzymes such as poly-ADP-ribose-polymerase (PARP) (*32*). A recent paper suggested that tectorigenin, one of the main components of the rhizome of *Iris tectorum* (*33*), induces apoptosis of HepG2 cells mainly *via* the mitochondrial-mediated pathway (*17*). The apoptosis of HepG2 cells was correlated with the production of reactive oxygen species (ROS), increased intracellular [Ca^{2+}], abnormal change of mitochondrial membrane potential, translocation of cytochrome *c*, activation of caspases-9, -8, and -3, and up-regulated transcription of endonuclease G and AIF-related genes in nuclear (*17*). Similar to this report, other studies also suggested the polyphenolic extract, galangin, genistein, and quercetin could induce apoptosis of HepG2 cells *via* changes of ROS and mitochondrial disruption (*18*,*34-36*). Moreover, JNK also play a critical role in a JNK-mediated apoptosis as well as mitochondrial-mediated (*37*). A study showed that 8-bromo-7-methoxychrysin (BrMC) promoted accumulation of intracellular ROS, initiation of caspase-3, and persistently activation of JNK in apoptosis of HepG2 and that, in JNK inhibitor-treated cells, BrMC-mediated apoptosis was partially attenuated (*38*). These suggest that the JNK pathway involves in BrMC-induced apoptosis of HepG2.

3.3 *Epidermal growth factor receptor (EGFR)/c-Met signaling pathway*

c-Met is frequently coexpressed with EGFR family members in human tumors, and it has been demonstrated that these receptor tyrosine kinases (RTKs) can crosstalk to each other and strengthen tumor cell invasion (*39-41*). In the next step, EGFR/c-Met signaling pathway can induce cancer cells proliferation, invasion, and angiogenesis through downstream molecules such as Ras, mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and Akt and so on (*42-45*).

It was reported that exogenous prostaglandin E2 (PGE2) stimulates cancer cell invasion through an intricate signaling axis requiring EGFR ligand production and c-Met (*46*). On the other hand, (−)-epigallocatechin-3-gallate (EGCG), one of the amplest bioactive constituents in leaves of green tea, was shown to inhibit HepG2 cell invasion *via* suppressing expression of PGE2 receptor EP1 through activation of EGFR/c-Met signaling (*47*). Besides, treatment of HepG2 cells with EGCG initiated apoptosis and led to a decrease in the phosphorylated insulin-like growth factor (IGF)-1 receptor protein and its downstream signaling elements including the p-ERK (extracellular signal-regulated kinase), p-Akt, p-Stat-3, and p-GSK3B (glycogen synthase kinase 3β) proteins. EGCG also decreased the levels of both IGF-1 and IGF-2 proteins, but increased the levels of the IGFBP-3 (insulin-like growth factor binding protein 3) protein. So, EGCG was considered to be an inhibitor of RTKs (*48*).

3.4 *NF-κB-related pathway*

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls the transcription of DNA and incorrect regulation of NF-κB is known to be linked to cancers (*49*). Therefore, NF-κB is expected as a target molecule in cancer therapy (*49*). Tumor necrosis factor α (TNF) plays an important role in initiating and perpetuating NF-κB signaling. TNF causes the activation of inhibitor of κB (IκB) kinase (IKK), which in turn phosphorylates and degrades inhibitor kappa B protein α (IκBα) and leads to NF-κB translocation to the nucleus and binding to a specific DNA consensus sequence; all this results in the transcriptional activation of NF-κB regulated genes involved in inflammation, such as cyclooxygenase-2 (COX-2) (*50*). There are reports that HCC can evade apoptosis by a common strategy of NF-κB activation which plays a role of adaptive resistance to apoptosis (*51*) and activates the pro-inflammatory chemokine interleukin (IL)-8 that promotes the progression of HCC (*52*). Xanthohumol, the major prenylated chalcone found in hops, was reported to have anti-HCC activity in NF-κB inhibition (*53*). Thus xanthomol can inhibit HepG2 cell proliferation *via* blocking tumor necrosis factor (TNF)-induced NF-κB activity in HCC cells *in vitro* and not affect viability of normal cells even in ten-fold higher concentration in comparison to that inhibiting HepG2 cells (*53*). Quercetin, a dietary flavonoid, has been shown to have anti-inflammatory effects through the downregulation of the NF-κB pathway (*54*). In a study in HepG2 cells, quercetin was demonstrated to suppress TNF-induced inflammation through downregulation of NF-κB, ERK, JNK, COX-2, and ROS generation (*55*). There are also other flavonoids which were reported to have NF-κB inhibition activity, such as a synthesized flavonoid LYG-202 (*56*), luteolin (*57*), epicatechin (*58*), and hesperidin (*59*).

3.5. *Heat shock protein (Hsp)-related pathway*

Heat shock proteins (HSP) are a class of functionally related proteins involved in the folding and unfolding of other proteins (*60*). Heat shock proteins function as intracellular chaperones for other proteins and monitor cell situation so as to initiate repair mechanism in time (*61*,*62*). As HSP acts as survival factors in cells, targeting HSP will be a new strategy for

cancer treatment (*63*,*64*). A flavonol constituent, quercetin, picked a diverse way to suppress tumor cell proliferation. For example, increased expression of Hsp27 and Hsp40 has been implicated in development of resistance to chemotherapeutic drugs by increasing DNA repair capacity, whereas quercetin is shown to potentiate chemotherapeutics by inhibiting the expression of Hsp27 and Hsp40 (*36*). An another research using SILAC (stable isotope labeling by amino acids in cell culture)-MS (mass spectrometry) assay, which is a technique to quantify the changes of whole protein spectrum, showed that quercetin can significantly suppress HepG2 cell's proliferation *via* inhibiting the expression of HSP in the cells (*65*).

3.6 *Tumor suppressor-related pathway*

A tumor suppressor gene, or anti-oncogene, is a gene that protects a cell from transforming to cancer cell. Tumor-suppressor gene-coding proteins repressively regulate the cell cycle and/or promote apoptosis (*66*). Activation of tumor suppressors could be a significant strategy for inhibiting tumor. Baicalein and silymarin, extract of *Scutellaria baicalensis*, have been reported to have a synergetic anti-tumor effect. They suppressed HepG2 cell proliferation by increasing the ratio of cells in the G0/G1 phase and decreasing those in S-phase, which were associated with up-regulation of tumor suppressors such as Rb, p53, p21Cip1, and p27Kip1 and down-regulation of cyclin D1, cyclin E, CDK4, and phospho-Rb (*67*). A recent study showed that diosmetin and luteolin, extracted respectively from *Rosa agrestis* Savi and artichoke (*Cynara scolymus*), could caused G2/M phase arrest in HepG2 and up-regulation of p53 and p21 proteins *via* CYP1A-catalyzed metabolism which always play role in scavenging chemical carcinogens (*68*).

Tumor cells always try their best to survive through evading apoptosis or promoting proliferation, invasion, and metastasis. Thus, understanding mechanisms of cancer cells survival pathways and flavonoids anti-tumor pathways will benefit anti-HCC medicines selection and therapy design.

4. Anti-HBV/HCV flavonoids

HCC is one of the most common and pernicious malignancies. More than 80% of HCC patients in worldwide obtain chronic hepatitis B and C infections. In China, approximately 95% of HCC patients have chronic HBV infection (*69*). Thus, in another aspect of HCC treatment, anti-virus medicines are also in an emergent need. Recently, various compounds including flavonoids isolated from herbs have been identified to possess anti-HBV or anti-HCV activity (*6*). There was a research report that in HepG2.117, a HBV inducible HepG2 cell line, EGCG suppressed HBV replication by spoiling HBV replicative intermediates of DNA synthesis, resulting in a decreased production of HBV DNA (*70*). In a recent study, Wogonin and 5-methoxy-(3,4"-dihydro-3",4"-diacetoxy)-2",2'-dimethylpyrano-(7,8:5",6")-flavone decreased the expression level of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) proteins and replication level of HBV DNA (*71,72*). In regard to HCV inhibition, catechin, isolated from TCM San-Huang-Xie-Xin-Tang, was identified to cause suppression of HCV replication and lead to a concentration- dependent down-regulation of COX-2 and NF-κB which have particular relevance to HCV-related HCC (*73*). Ladanein was reported to suppress a post-attachment entry progression, rather than RNA replication or HCV assembly and effectively resist major HCV genotypes, including a variant which is resistant to an entry inhibitor (*74*).

As one of the most fatal cancers, especially in East Asia, more and more attention focuses on the treatment on HCC. Owing to the anti-tumor and anti-virus effect of some TCM and other herb medicines, the flavonoids extracted from TCM and other herbs were regarded as ideal candidates for HCC therapy. With further study, a many flavonoids showed anti-HCC or anti-HBV/HCV activity in experiments *in vitro* and/or *in vivo*. Some research data suggested the combination therapy of one flavonoid with other flavonoids or chemotherapeutics could greatly enhance efficiency. Furthermore, there will be more flavonoid medicines being developed and more therapies emerging.

5. Conclusion

In conclusion, flavonoids extracted from TCM and other herb medicine has shown interesting anti-tumor activity on various cancer cells including HCC. More and more flavonoids are continuing to be isolated from TCM and other herbs, which provides a tremendous pool for effective compound screening. Modern molecular biological technology and cell biological technology accelerate the screening. The accumulating effective flavonoids acting on diverse cellular signaling pathways make it possible to optimize the therapy by new medicine alteration and combination of two or more medicines. Based on former data, combination therapy has exhibited higher effectiveness than single drug therapy. Owning to a reality that some flavonoids-rich TCMs are fruits and vegetables, the combination of clinical therapy and planned dietetic therapy may obtain more satisfying results. At present, translating flavonoids into clinical medicines is a major mission for medical researchers.

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Review

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Derivatization in liquid chromatography for mass spectrometric detection

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ABSTRACT: Liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) has been frequently utilized for the sensitive and selective determination of the trace level compounds in biological samples. In LC/ESI-MS/MS, chemical derivatization is sometimes used to enhance the detection sensitivity of the analytes. This review presents an overview of the derivatization reagents in LC/ESI-MS/MS that have been applied to the low molecular weight compounds in recent five years (2008-2012).

Keywords: Liquid chromatography, electrospray, tandem mass spectrometry, derivatization

1. Introduction

Liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) has been frequently utilized for the sensitive and selective determination of the trace level compounds in biological samples. In particular, LC/MS/MS equipped with electrospray ionization (ESI) as the ion source is most often used method, since ESI can ionize wide range of the compounds including the polar compounds or large molecular weight compounds. Furthermore, ESI requires lower temperature for ionization compared with other ionization methods such as atmospheric pressure chemical ionization (APCI), and thus it can be used for the thermally unstable compounds.

In LC/ESI-MS/MS, the analytes having the following properties can be sensitively detected. Firstly, the analytes must be ionic or ionizable forms in the solution phase, since, in the ESI, gas phase ions are mainly generated by transferring the ions in solution into gas phase in the presence of a strong electrical field. Secondly, it is preferable for the analytes to have the appropriate hydrophobic structures, since (*i*) the hydrophobic ions prefer to reside at the droplet surface generated by electrospray and these ions enter the gas phase more readily than those in the droplet interior and show the higher signal intensities. (*ii*) The hydrophobic compounds can be well separated on the reversed phase column from salts and interfering compounds possessing suppression effects on ESI. (*iii*) The hydrophobic compounds are eluted by the mobile phase with the higher organic solvent content. The higher organic solvent content is suitable for the stable generation of charged droplets by electrospray and thus gives the higher signal intensities. Thirdly, it is desired for the analytes to have the suitable structure for MS/MS detection (selected reaction monitoring (SRM)), *i.e.*, to fragment efficiently upon collision induced dissociation (CID) and generate an intense and particular product ion. However, not all the compounds can be favorably analyzed by LC/ESI-MS/MS. Thus, chemical derivatization of the analyte is often used to enhance the detection sensitivity.

So far, a number of derivatization reagents for LC/MS/MS have been reported and they are summarized in several review papers (*1-7*). This review presents an overview of the derivatization reagents for LC/ESI-MS/MS that have been applied to low molecular weight compounds in biological samples in recent five years (2008-2012).

2. Derivatization reagents and their application

It is preferable for the derivatization reagents in LC/ESI-MS/MS to have the following properties. (*i*) The reagent reacts with the analytes under the mild conditions and produces the derivatives in high yield. Usually the reagent having a small molecular weight is favorable for this purpose. The reagent generates the derivatives having (*ii*) the ionic or ionizable moieties, (*iii*) the appropriate hydrophobicity, and (*iv*) the suitable structures for SRM. (*v*) The reagent is commercially available or can be easily synthesized.

In the early stage, the derivatization reagents were used mainly for the improvement of the chargeability. Quirke *et al.* reported the derivatization of alkyl halides, alcohols, phenols, thiols, and amines using a number of

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the reagents to achieve the enhancement of the signal intensities. A typical example is the derivatization of alcohol using 2-fluoro-1-methylpiridinium *p*-toluensulfonate. Cholesterol was converted to its *N*-methylpiridyl ether, and an intense [M^+] ion of the derivative was observed in positive ESI-MS (*8*). To introduce a permanently charged moiety is sometimes called "charged derivatization" and to introduce an ionizable moiety is called "ionizable derivatization". Usually, charged derivatization gives the stronger signal intensity in ESI-MS, however, the compounds having permanently charged moieties were not always suitable for the separation on the reversed phase column. To improve the hydrophobicity is also effective to enhance the detectability, since the analytes' affinity for the droplet surface (surface activity) strongly affects the ESI response. Nordstrom *et al.* used propionyl and benzoyl anhydride for the derivatization of bases, cytokinins, ribosides, and intact nucleotides such as AMP, ADP, and ATP (*9*). The ESI response was enhanced by the formation of hydrophobic derivatives and the retention on a reversed phase column was greatly increased. Tandem mass spectrometry (MS/MS) enables sensitive detection of the analytes, since MS/MS detection decreases the noise level and improves the signal-to-noise ratios comparing with MS detection. Therefore, the transformation of the analyte to the structure suitable for SRM is effective to enhance the sensitivity. Usually, esters, hydrazones, urea and thiourea, aromatic sulfonyl compounds, amides, and alkyl quaternary ammonium compounds are efficiently fragmented upon CID and generate an intense and particular product ion. Recently, commercially available reagents are favorably utilized for this purpose.

2.1. *Alcohols and phenols*

Alcohols and phenols are neutral compounds and they are sometimes too hydrophilic. Therefore, deivatization is used to enhance the chargeability or hydrophobicity of these compounds. One of the most widely used reagents is dansyl chloride (5-dimethylamino-1-naphthalenesulfonyl chloride; Dns-Cl). Dns-Cl has a tertiary amino group as an ionization moiety and an aromatic ring as a hydrophobic moiety. And Dns-Cl generates the aromatic sulfonyl compounds which are suitable for SRM. Chang *et al.* used Dns-Cl for simultaneous quantification of multiple classes of 30 phenolic compounds, including estron, α-estradiol, β-estradiol, ethinylestradiol, and bisphenol A, in blood (*10*). After extraction, the phenolic compounds were reacted with Dns-Cl at 60ºC for 5 min in acetone and sodium bicarbonate buffer (pH 10.5). The generated derivatives were separated on the reversed phase column and detected upon ESI-MS/MS. As expected, protonated molecules ([M + H]$^+$) of their dansyl derivatives were observed in the mass spectra, and the common major product ions at *m/z* 171 and 156 were observed in the product ion spectra. The ion at *m/z* 171 originated from a cleavage of a C-S bond in dansyl portion of the molecule, and the ion at *m/z* 156 was produced by loss of the methyl group from the ion at *m/z* 171. The transitions from protonated molecules to *m/z* 171 and *m/z* 156 were monitored and these derivatives were sensitively detected. The attained detection limit of bisphenol A was 180-fold higher compared with the direct LC/MS analysis of the intact compound. Dns-Cl was also used for the derivatization of the alcohols, including testosterone, cholesterol, hydrocortisone, *etc*. Dns-Cl reacted these compounds at 60ºC for 1 h in the presence of 4-(dimethylamino)-pyridine and *N*,*N*-diisopropylethylamine. The generated derivatives were sensitively analyzed by LC/ESI-MS/MS (*11*). Dns-Cl is widely used for the analysis of the compounds having hydroxyl group (*10-19*). Similarly, picolinic acid (*20-26*), fusaric acid (*27*), dimethylglycine (*28*), isonicotinoyl azide (*29,30*), and *N*-methyl-nicotinic acid *N*-hydroxysuccinimide ester (C1-NA-NHS) (*31*) were used for the derivatization of alcohols and phenols. These reagents have an ionization moiety and a hydrophobic moiety, and the generated derivatives were efficiently fragmented upon CID and were suitable for SRM. The derivatization reagents for alcohols and phenols are summarized in Table 1.

2.2. *Aldehydes and ketones*

Aldehydes and ketones are neutral compounds and the ionization efficiencies in ESI of these compounds are sometimes low. Therefore a chargeable moiety is introduced to enhance the ionization efficiency. In the early stage, hydroxylamine, 2,4-dinitrophenylhydrazine (DNPH), 1-(carboxymethyl)pyridium chloride hydrazide (Girard's P), and (carboxymethyl)trimethylammon ium chloride hydrazide (Girard's T) were used for derivatization of carbonyl compounds. These reagents enhanced the ionization efficiency of aldehydes and ketones. However, usually, several product ions were observed in the product ion spectra of the generated derivatives. Therefore they were not always suitable for the sensitive detection by SRM, though these product ions were structurally informative. The promising reagents for carbonyl compounds are 2-hydrazino-1-methylpyridine (HMP) and 2-hydrazinopyridine (HP). HMP has a quaternary ammonium group as an ionization moiety and a hydrophobic aromatic structure. Shibayama *et al.* used HMP for the analysis of oxo-steroids such as testosterone (T) and dehydroepiandrosterone (DHEA) in saliva (*36*). HMP reacted with these compounds at 60ºC within 1 h in ethanol containing 0.5% trifluoroacetic acid (TFA). The derivatives provided intense [M^+] ions in MS analysis. And in the MS/MS analysis, the product ion at *m/z* 108 (T, [*N*-methylpyridine + NH^+]) or *m/z* 109 (DHEA, [*N*-methylpyridine + NH_2^+]) were observed, which

were formed by the cleavage of the N-N bond of the hydrazone. The HMP-oxo-steroids were separated on the reversed phase column and detected by ESI-MS/MS. The sensitivity of DHEA was 2,000 times higher compared with the analysis without derivatization. However, HMP is not effective for increasing the detection responses of di-oxo-steroids, since small molecules with a multi-charge are unstable in the gas phase and provided multiple ions. To overcome these problems, HP was used for di-oxo-steroids such as 17α-hydroxyprogesterone (17OHP) (*38*). HP does not have a permanently charged moiety and thus the generated derivative does not form a multiply charged compound. HP is usable for both mono- and di-oxo-steroids (*39,40*). Dansyl hydrazine (*41*), 4-(2-(trimethylam monio)ethoxy)benzenaminium dibromide (4-APC) (*49*), 4-(2-((4-bromophenethyl)dimethylammonio)ethoxy)benze naminium dibromide (4-APEBA) (*50*), were also used for the derivatization of aldehydes and ketones. These reagents have an ionization moiety and a hydrophobic moiety, and produced the derivatives having the suitable structure for SRM. The derivatization reagents for aldehydes and ketones are summarized in Table 2.

Table 1. Derivatization reagents for alcohols and phenols

Derivatization reagent	Analyte (sample) (*reference*)
Dns-Cl	Multiple class of 30 phenols (blood) (*10*); hydroxysterols, retinol, cholecalciferol, 12-OH dodecanoic acid, 3-OH palmitic acid, *etc*) (tissue extracts) (*11*); 17β-estradiol (brain) (*12*); estrone, estradiol (serum) (*13*); estrone, estradiol, estriol, and their metabolites (commercial milk products) (*14*); eight steroids (androgen, oestrogen, progestogen) (food production) (*15*); 4-dimethylaminophenol (blood) (*16*); morphine (urine) (*17*); estradiol (serum) (*18*); estrogen, estrogen metabolites (cell culture) (*19*).
Picolinic acid	19 Cholesterol precursors, 11 available sterols (*20*); 4β-hydroxycholesterol, 7α-hydroxycholesterol, 22R-hydroxycholesterol, 24S-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, 24S,25-epoxycholesterol (serum, rat liver microsomes) (*21*); aldosterone (standard) (*22*), aldosterone, dehydrocorticosterone, corticosterone, cortexolone (serum) (*23*); tetrahydrocortisol, allotetrahydrocortisol, tetrahydrocortisone (urine) (*24*); 2-hydroxyestrone, 2-hydroxyestradiol, 4-hydroxyestrone, 4-hydroxyestradiol (incubation mixture) (*25*); testosterone, dihydrotestosterone (serum, prostate tissue) (*26*).
Fusaric acid	Dehydroepiandrosterone, testosterone, pregnenolone, 17α-OH-pregnenolone (standard) (*27*).
Dimethylglycine	Cholesterol oxidation products (plasma) (*28*).
Isonicotinoyl azide	Androgens, androsterone, 5α-androstane-3α,17β-diol (brain and serum) (*29*); 5α-androstane-3α,17β-diol (brain, serum) (*30*).
C1-NA-NHS	16 Estrogens (serum) (*31*).
FMP	Dihydrotestosterone (prostatic tissue) (*32*); propofol (blood, serum) (*33*); dihydrotestosterone, androstanediol, androstanediol-glucronide (prostate tissue) (*34*).
3-Nitrophtalic anhydride	Isoprenols (farnesol, geranylgeraniol) (tissue) (*35*).

Dns-Cl, 5-dimethylamino-1-naphthalenesulfonyl chloride; FMP, 2-fluoro-1-methylpyridinium *p*-toluenesulfonate; C1-NA-NHS, *N*-methyl-nicotinic acid *N*-hydroxysuccinimide ester.

Table 2. Derivatization reagents for aldehydes and ketones

Derivatization reagent	Analyte (sample) (*reference*)
HMP	Testosterone (saliva) (*36*); allopregnanolone (brain) (*37*).
HP	17α-Hydroxyprogesterone (saliva) (*38*); 17α-hydroxypregnenolone, progesterone, 11-ketotestosterone, 11-deoxycortisol, 17α, 20β-dihydroxypregnenone (plasma) (*39*); 17α-hydroxypregnenolone and 17α-hydroxyprogesterone (blood spots) (*40*).
Dns-Hz	Malondialdehydes (plasma) (*41*).
Girard's P	3-Oxosterols (profiling) (blood) (*42*).
Girard's T	Oxysterols (24-hydroxycholesterol, 25-hydroxycholesterol, 22-hydroxycholesterol) (cell lysate) (*43*).
DNPH	Malonedialdehyde, 4-hydroxynonenal (exhaled breath condensate) (*44*); malonedialdehyde (urine) (*45*).
Hydroxylamine	17α-Hydroxypregnenolone, 17α-hydroxyprogesterone, androstenedione, dehydroepiandrosterone, testosterone, pregnenolone, progesterone (serum) (*46*); androstenodione, dehydroepiandrosterone, testosterone (serum) (*47*).
DAABD-MHz	Aliphatic aldehydes (standard) (*48*).
4-APC	Aldehydes (malondialdehydes, aliphatic aldehydes (C5-C10)), ketones (urine) (*49*).
4-APEBA	Aliphatic aldehydes (C5-C10) (urine and plasma) (*50*).
D-Cysteine	Aliphatic aldehydes (C1-C8) (beverages) (*51*).
HTP	Testosterone, dihydrotestosterone (tissue) (*52*).

HMP, 2-hydrazino-1-methylpyridine; HP, 2-hydrazinopyridine; Dns-Hz, 5-dimethylaminonaphthalene-1-sulfonyl hydrazine; Girard's P, 1-(carbo xymethyl)pyridium chloride hydrazide; Girard's T, (carboxymethyl)trimethylammonium chloride hydrazide; DNPH, 2,4-dinitrophenylhydrazine; DAABD-MHz, 4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-*N*-methylhydrazino-2,1,3-benzoxadiazole; 4-APC, 4-(2-(trimethylammonio)-ethoxy)benzenaminium halide; 4-APEBA, 4-(2-((4-bromophenethyl)dimethylammonio)ethoxy)benzenaminium dibromide; HTP, 2-hydrazino-4-(trifluoromethyl)-pyrimidine.

2.3. *Carboxylic acids*

Carboxylic acids are detectable in the negative ESI-MS. However their sensitivities are rather poor. In addition the mobile phases for the carboxylic acids separation are not always compatible with ESI-MS. Therefore carboxylic acids are sometimes transformed to the hydrophobic and ionizable structures. Higashi *et al.* reported the simple and practical method for the analysis of carboxylic acids using 2-hydrazinopyridine (HP) (*53*) and 2-picolylamine (PA) (*54*). Several biological important compounds having carboxylic acids, such as chenodeoxycholic acid, were derivatized with these reagents at 60ºC for 10 min in the presence of the condensation reagents. The resulting HP- and PA-derivatives were highly responsive in ESI-MS. And in MS/MS analysis, the derivatives of HP gave the strong product ion at *m/z* 110, derived from the protonated 2-hydrazinopyridine moiety, and those of PA gave the strong product ion at *m/z* 109, derived from 2-picolylamine moiety. The transitions to *m/z* 109 from the $[M + H]^+$ of the PA-derivatives were used for SRM. The PA-derivatization was successfully applied to a biological sample analysis. The derivatization followed by LC/ESI-MS/MS enabled the detection of trace amount of the clinically important carboxylic acids such as bile acids, homovanillic acid in human saliva. The detection responses of PA-derivatives were increased by 9-158 folds over the intact carboxylic acids (*54*). Similarly, 3-picolylamine, 3-picolylcarbinol (*55*), 3-hydroxy-1-methyl-piperidine (*56*), 3-(hydroxymethyl)-pyridine (*57*), 4-APEBA (*50*,*58*), 4-diazomethylpyridine (*59*), and 4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole (DAABD-AE) (*60*,*61*) were used for the derivatization of carboxylic acids. These reagents have an amino group as ionization moiety and a hydrophobic moiety. The formed derivatives, such as esters or amides, were efficiently fragmented upon CID and were suitable for SRM. Butanol-HCl was sometimes used for the derivatization of carboxylic acids (*62*-*65*). The generated butyl esters were rather hydrophobic and often gave the characteristic product ions. Butanol-HCl derivatization is routinely used for the simultaneous analysis of amino acids and acylcarnitines in dried blood spots for newborn screening. Amino acids and acylcarnitines themselves have the ionizable amino groups. Therefore, the improvement of the hydrophobicity and formation of the suitable structure for MS/MS detection are required to enhance the detectability. A simple butanol-HCl derivatization is suitable for this purpose. Benzyl alcohol (*66*) and 9-fluorenylmethyl chlorformate (FMOC-Cl) (*67*) also improved hydrophobicity of the analytes and produced the suitable structures for SRM. The derivatization reagents for carboxylic acids are summarized in Table 3.

2.4. *Amines*

The compounds having amino group are easily protonated under acidic conditions and suitable for ESI-MS. However, they are sometimes highly polar and too hydrophilic for the separation on the reversed

Table 3. Derivatization reagents for carboxylic acids

Derivatization reagent	Analyte (sample) (*reference*)
2-Hydrazinopyridine (HP)	Chenodeoxycholic acid and glycochenodeoxycholic acid (saliva) (*53*).
2-Picolylamine (PA)	Chenodeoxycholic acid, glycochenodeoxycholic acid, prostaglandin E_2, 2-(β-carboxyethyl)-6-hydroxy-2,7,8-trimethylchroman, α-lipoic acid, homovanillic acid, 5-hydroxyindole-3-acetic acid) (saliva) (*54*).
3-Picolylamine, 3-picolylcarbinol	Fatty acids (C16-C28) (red blood cell) (*55*).
3-Hydroxy-1-methyl-piperidine	Malonic acid (serum) (*56*).
3-(Hydroxymethyl)-pyridine	Aliphatic molecules containing carboxylic acids such as jasmonic acid and salicylic acid (plant extracts) (*57*).
4-APEBA	Aliphatic acid (C5-C9) (urine, plasma) (*50*); hexanoic acid, heptanoic acid, octanoic acid, nonanoic acid, hippuric acid, benzoic acid, hydrochloric acid, prostaglandins PGE1, PGE2, PGF2α, ibuprofen, ketoprofen, naprofen, *etc*. (urine) (*58*).
4-Diazomethylpyridine	Prostaglandin E2 (brain) (*59*).
DAABD-AE	Very long chain fatty acids (C19, C20, C22, C24, C26), phytanic acid, pristanicacid (plasma) (*60*); glutaric acid, 3-hydroxyglutaric acid (dried urine spots) (*61*).
Butanol + HCl	Succinylacetone (dried blood spots) (*62*); 3-aminolevulinic acid and porphobilinogen (urine, plasma) (*63*); guanidinoacetate (plasma) (*64*); 22 amino acids (plasma, serum) (*65*).
Benzyl alcohol + HCl	Low molecular organic acids (citric acid, fumaric acid, malic acid, malonic acid, oxalic acid, succinic acid, acotinic acid) (root exudation) (*66*).
FMOC-Cl	Valproic acid (serum) (*67*).
N-Methyl-2-phenylethanamide	Di- and tri-carboxylic acids (tricarboxylic acid cycle intermediates) (heart tissue) (*68*).
p-Dimethylaminophenacyl bromide	Profiling carboxylic acid containing metabolites (urine) (*69*).

4-APEBA, 4-(2-((4-bromophenethyl)dimethylammonio)ethoxy)benzenaminium dibromide; DAABD-AE, 4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3- benzoxadiazole; FMOC-Cl, 9-fluorenylmethyl chlorformate.

phase column. These compounds are transformed to more hydrophobic structures by derivatization. Furthermore, the increase in the molecular weight decreases in the background noise from the matrix, since the background is generally lower in the higher mass range. *p*-*N*,*N*,*N*-Trimethylammonioanilyl-*N*'-hydroxysuccidimidyl carbamate iodide (THAS) (*70*) and 3-aminopyridyl-*N*-hydroxysuccimidyl carbamate (APDS) (*71*) were the reagents designed for LC/ESI-MS/MS. THAS has a quaternary ammonium group as an ionization moiety and a hydrophobic aromatic structure. It reacted with amino acids at 60ºC for 10 min in borate buffer (pH 8.8) to form urea compounds. The urea bond of the derivatives was efficiently cleaved upon CID, and produced the particular product ion at *m/z* 177, derived from the reagent moiety. Amino acids were analyzed with the detection limits of atto-mole level (*70*). APDS has a more hydrophobic structure compared with THAS, and thus APDS derivatives are suitable for the separation on the reversed phase column. APDS was used for the analysis of amino acids (*72*,*73*) and more than 100 compounds having amino group in biological fluid. The transition of all the protonated molecules to the common product ion at *m/z* 121 was monitored (*71*). As described above, urea and thiourea moieties are efficiently fragmented upon CID and they are suitable for SRM. 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), the commonly used fluorescence derivatization reagent for amines, also generated the derivatives having urea structure and thus suitable for SRM. It was applied to the analysis of amino acids (*74*) and peptides (*75*,*76*). The derivatives gave the characteristic product ions at *m/z* 145 and *m/z* 171 derived from the AQC moiety (*75*). Dns-Cl (*77*,*79*), 3-pyridyl isothiocyanate (*80*), 4-methylpiperazinebutyl succimide (MPBS) (*81*), and dimethylaminobutyl succimide (DMABS) (*81*) have an ionization moiety and a hydrophobic moiety, and generated the suitable structures for SRM. Phenyl isothiocyanate (*83*), propyl chloroformate (*84*), 9-fluorenylmethyl chloroformate (FMOC) (*84*,*85*), and isobutyl chloroformate (*86*) have a hydrophobic moiety and generated suitable structure for SRM, though these reagents do not have the strong ionization moiety such as amino group. The derivatization reagents for amines are summarized in Table 4.

3. Conclusion

The derivatization reagents in LC/ESI-MS/MS applied to the low molecular weight compounds are summarized. These reagents enhanced the chargeability and hydrophobicity of the analyte to improve the ionization efficiency. These reagents also transformed the analytes to the structures suitable for SRM. So far, a number of compounds were used as the derivatization reagents in LC/ESI-MS/MS. It is important to utilize the suitable reagent for the target analytes. Furthermore,

Table 4. Derivatization reagents for amines

Derivatization reagent	Analyte (sample) (*reference*)
THAS	Amino acids (plasma) (*70*).
APDS	100 Compounds with amino group (plasma) (*71*); amino acids (plasma) (*72*,*73*).
AQC	Amino acid profiles (tear fluids) (*74*); γ-Glu-Val-Gly (fish sauces) (*75*); β-*N*-methylamino-L-alanine and 2,3-diaminobutylic acid (various biological samples) (*76*).
Dns-Cl	α-Fluoro-β-alanine, 5-fluorourasil, capecitabine (plasma) (77); gemcitabine and 2,2-difluoro-2-deoxyuridine (plasma) (*78*); 672 metabolites containing primary amine, secondary amine, phenolic hydroxyl group (urine) (*79*).
3-Pyridyl isothiocyanate	Aliphatic amines (C8-C16) (standard) (*80*).
MPBS, DMABS	Amino acids (plasma, blood spots) (*81*).
iTRAQ reagent	Amino acids (urine) (*82*).
Phenyl isothiocyanate	Glycine (cerebrospinal fluid) (*83*).
Propyl chloroformate	Amino acids (microdialysis sample) (*84*).
FMOC-Cl	Amino acids (standard) (*84*); glyphosate and its aminomethylphosphoric acid (cereals) (*85*).
Isobutyl chloroformate	L-prolyl-4-L-hydroxyproline (urine) (*86*).
SPTPP	Amines and amino acids (serum) (*87*).
o-Phthaldialdehyde + thiol	Arginases inhibitor, arginine (plasma) (*88*); glucosamine (urine) (*89*); dimethylarginines (plasma) (*90*).
DBD-F	Nine polyamines (nail) (*91*).
p-Bromophenacyl bromide	Anticancer chemicals (tegafur, 5-fluorouracil, gimeracil) (urine, plasma) (*92*).
Ethyl bromoacetate	Trimethylamine (urine) (*93*).

THAS, *p*-*N*,*N*,*N*-trimethylammonioanilyl-*N*'-hydroxysuccidimidyl carbamate iodide; APDS, 3-aminopyridyl-*N*-hydroxysuccimidyl carbamate; AQC, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate; Dns-Cl, 5-dimethylamino-1-naphthalenesulfonyl chloride; iTRAQ, isobaric tags for relative and absolute quantification; MPBS, 4-methylpiperazinebutyl succimide; DMABS, dimethylaminobutyl succimide; SPTPP, (5-*N*-succinimidoxy-5-oxopententyl)triphenylphosphonium bromide; DBD-F, 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole; FMOC-Cl, 9-fluorenylmethyl chloroformate.

it might be also helpful to adopt the methods such as mobile phase modification, hydrophilic interaction chromatography, or nano-electrospray to enhance the detectability of the various kinds of the analytes in the fields of biomedical analysis.

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Brief Report

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The ethanol extract of *Cirsium japonicum* increased chloride ion influx through stimulating $GABA_A$ receptor in human neuroblastoma cells and exhibited anxiolytic-like effects in mice

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ABSTRACT: The aim of the present study was to evaluate the anxiolytic effects of the ethanol extract of *Cirsium japonicum* (CJ) in mice. The extract was orally administered at dosages of 50, 100, 200, or 400 mg/kg of body weight. The CJ-induced behavioral changes were assessed using the open-field and elevated-plus maze test. The ethanol extract of CJ did not affect overall locomotor activity of mice in the open-field test, however, it showed increase exploration in the unprotected center zone, which is thought to reflect anxiolytic-like effects. Furthermore, the CJ extract (100 and 200 mg/kg) significantly increased the percentage of time spent in the open arms of the elevated plus-maze, indicating the anxiolytic effects of the substance. This anxiolytic effects of the extract were comparable to that of the benzodiazepine, diazepam. To further characterize the anxiolytic activities of CJ, its action on human neuroblastoma cells were assessed. The CJ extract dose-dependently increased chloride ion (Cl^-) influx, which was blocked by co-administration of the $GABA_A$ receptor competitive antagonist, bicuculline, suggesting a $GABA_A$ receptor – Cl^- channel mechanism of action. Taken altogether, the present study demonstrates that the ethanol extract of CJ has anxiolytic effects, probably mediated through GABAergic neurotransmission.

Keywords: *Cirsium japonicum*, anxiolytic, benzodiazepine, GABA, anxioselective

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1. Introduction

Anxiety is a psychological and physiological condition characterized by emotional, cognitive, somatic, and behavioral factors. It is considered as normal reaction to stressors and is important for survival, since it alerts us to danger and prepares us to cope with imminent situations (*1*). However, when anxiety is overwhelming, it may fall under the classification of anxiety disorder. Anxiety disorders are the most common psychiatric diagnosis in general population which has an estimated lifetime prevalence of about 16% and a 12-month prevalence of 11% (*2*). Anxiolytics or anti-anxiety agents are drugs used to relieve anxiety and manage its related psychological and physical symptoms. Thus drugs usually act as depressors of the central nervous system (CNS) (*1,2*). Presently, benzodiazepines (*e.g.* diazepam) are one of the drugs of choice in treating/controlling anxiety. However, it is prescribed with special considerations due to its common side effects (*1*). With the hope of finding an alternative treatment for anxiety with fewer side effects, many researchers have delved into the study of herbal medicines or plant extracts (*3,4*). Currently, studies on herbal medicine as complementary and alternative medicine (CAM) for mild to moderate anxiety disorders are becoming popular and accepted worldwide (*4,5*).

Cirsium japonicum (CJ) is a perennial herb native to China, Japan, and Korea belonging to Compositae family (*6*). It has been classified in the Japanese and Chinese Pharmacopeia and was used in various preparations as an antihemorrhagic, antihypertensive, and uretic agent. CJ has also been traditionally used as an antioxidant, antidiabetic, and antimicrobial (*7-10*). There are several studies about the pharmacological effects of CJ, but very limited information is available about its psychopharmacological effects. A recent notable study with CJ shows that it has antidepressant property, causing significant decrease in depression associated with pre-menstrual syndrome (*11*).

Considering the above-mentioned effects, we decided to assess whether CJ has anxiolytic properties.

Thus, the goal of the present study was to evaluate the psychopharmacological activities of CJ, specifically its probable anxiolytic effect. The ethanol extract of CJ was dissolved in water and were orally administered to mice in dosages of 50, 100, 200, or 400 mg/kg of body weight. Then, behavioral changes consequential to CJ treatment were observed in the open-field and the elevated plus-maze test. The open-field test provides a unique opportunity to systematically assess novel environment exploration, general locomotor activity, and provide an initial screening for anxiety-related behavior, through exploration on the unprotected center area (*12*). Furthermore, CJ's effects were evaluated in the elevated plus-maze test, the most widely used and well-established paradigm in assessing anxiety-like behavior in rodents (*13*). The effect of one of the most commonly used and prescribed anxiolytic agent, the benzodiazepine diazepam, was also evaluated and used as a reference drug. Moreover, to further characterize the anxiolytic effects of CJ, its action on the γ-aminobutyric acid (GABA) receptor-chloride ion (Cl^-) channel complex was assessed in human neuroblastoma cells (*1,12,14*).

2. Materials and Methods

2.1. *Animals*

The male ICR mice (20-25 g) used in the present study were obtained from Hanlim Laboratory Animals Co. (Hwasung, Korea). They were housed in groups in a temperature-controlled (22 ± 2°C) and humidity-controlled (55 ± 5%) animal room on a 12/12 h light/dark (7:00-19:00 h light) schedule. Food and water were freely available, except the night before and during the experiments. Mice were allowed to acclimatize to the laboratory setting, for at least 7 days, before the commencement of any experiments. Eight to ten animals were used in each experimental group. Animal treatment and maintenance were carried out in accordance with the Principles of Laboratory Animal Care (NIH publication No. 85-23 revised 1985) and the Animal Care and Use Guidelines of Sahmyook University, Korea.

2.2. *Drugs and materials*

Samples of aerial part of CJ were obtained from Cheju province of Korea, and were examined by botanical experts. The dried sample (10 g dry weight) was subjected to ethanol extraction (900 mL) by shaking for 25 h at room temperature. The obtained ethanol extract was filtered then freeze-dried. Diazepam, bicuculline, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The CJ extract and diazepam were diluted with sterile distilled water before administration. *N*-(6-Methoxyquinolyl) acetoethylester (MQAE) was purchased from Invitrogen Co. (Carlsbad, CA, USA). Bicuculline was dissolved in DMSO, with a maximum concentration of 0.1%. CJ extract was orally administered to mice in dosages of 50, 100, 200, or 400 mg/kg. One mg/kg diazepam was intraperitoneally (*i.p.*) injected to mice belonging to the reference group. Animals in the control group received the vehicle (distilled water).

2.3. *Psychopharmacological evaluation*

2.3.1. *Open-field test*

Mice were placed in an open-field arena, consisting of a square plexiglas container (42 × 42 cm) with a field bordered by 42 cm high sidewalls. Mice were orally pre-treated with CJ, or vehicle, or diazepam (*i.p.*), 30 min before the commencement of the test. Prior to recording, animals were first habituated to the open-field for 2 min to remove the bias of novelty. The observed parameters were moved distance on open-field arena and unprotected center zone, was recorded for 10 min (*12*). Ethovision (Noldus, Netherlands) system was used to record animal movement.

2.3.2. *Elevated plus-maze (EPM) test*

The elevated plus-maze box and arms were made of plastic. The plus maze consisted of four arms that comprised of two open arms (30 × 6 cm in mice) and two closed arms (30 × 6 cm in mice) enclosed by 20 cm high walls. Each arm had a delimited central area of 6 × 6 cm. The entire maze was elevated to a height of 50 cm above the floor. Mice were orally pre-treated with CJ, vehicle or diazepam (*i.p.*), 30 min before placement on the EPM. To begin a test session, mice were placed in the center of the maze facing one of the open arms. An entry into an arm was defined as the animal placing all four paws over the line marking that area. The observed parameters were (*i*) time spent in the open arms and (*ii*) number of entries into the open arms during the 5 min test period (*13*). The percentage of open arm entries (100 × open/total entries) was calculated for each animal.

2.3.3. *Assay of Cl^- influx*

Human neuroblastoma SH-SY5Y cells (Korean Cell Line Bank, Korea) were cultured in minimum essential medium (MEM) (Invitrogen Co.) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich Co.) in a humidified incubator of 95% air and 5% CO_2 at 37°C.

Experiments were performed according to the methods of Yoon *et al*. (*15*). Briefly, cells were washed twice and suspended at a concentration of 4×10^5

cells/ ml in Hank's solution. For loading MQAE into the cells, cells were incubated with the dye overnight in a final concentration of 5 mM at room temperature. Fluorescence (excitation wavelength set as 365 nm, the emission wavelength at 450 nm) was monitored in a well-stirred cuvette. Experiments were performed at room temperature to minimize fluorescence dye loss. Data are presented as relative fluorescence F/F_0, where F_0 is minimize fluorescence without Cl^- ions and F is the fluorescence as a function of time. The F/F_0 was directly proportional to $[Cl^-]_i$. All fluorescence values were corrected for background fluorescence which was separately determined using HEPES-buffered KSCN solution containing 5 µM valinomycin to maximally quench the MQAE ion-selective signal. In separate experiments the F_0 value was determined by bathing the cells with Cl^--free (KNO_3) solution containing 10 mM tributyltin and 10 mM nigericin.

2.4. *Statistical analysis*

Data are expressed as mean ± the standard error of the mean (SEM). For statistical evaluation of data, one-way analysis of variance (ANOVA) was used. When statistically significant differences were found, Dunnett test was used as a post-hoc test, to determine the statistical difference between groups. Differenced were considered statistically significant when $p < 0.05$.

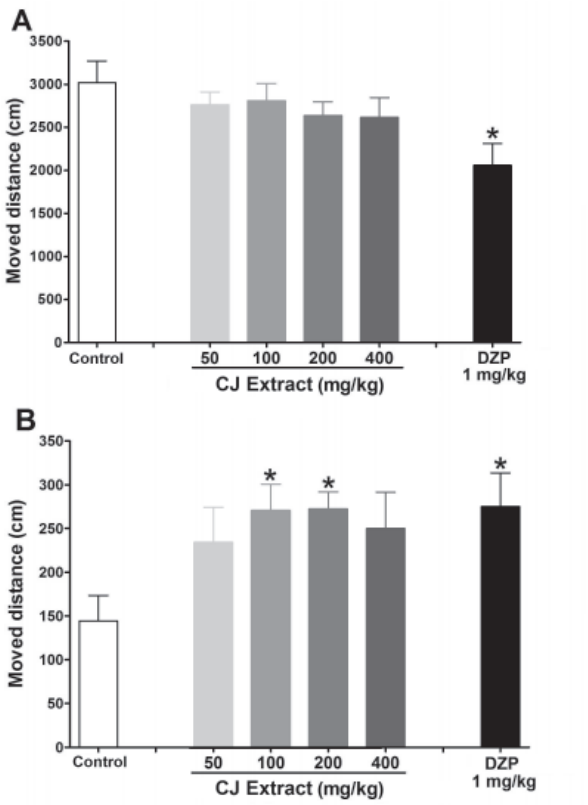

Figure 1. Effects of ethanol extract of CJ on locomotor activity in mice. Each bar represents the mean ± SEM (n = 8-10) of the moved distance in arena **(A)** and center zone **(B)**, for 10 min. * $p < 0.05$ significantly different from the control group. DZP, diazepam.

3. Results

3.1. *Open-field test*

Figure 1 shows the distance moved in the open-field arena and unprotected center area of animal treated with the CJ extract, diazepam, or vehicle (control group). Ambulatory activity was significantly decreased by diazepam ($q = 3.16$, $p < 0.05$) as compared to the control group, indicative of its sedative effects (*16*). On the other hand, the CJ extract did not significantly affect ambulatory activity in the open-field arena. In addition, diazepam significantly increased exploratory time at the unprotected center zone ($q = 2.84$, $p < 0.05$). This increase in exploration in the center area was thought to reflect the anxiolytic property of diazepam (*17*). Similarly, the CJ extract showed a significant increase in exploratory activity in the center area of the open-field test at doses of 100 ($q = 2.75$, $p < 0.05$) and 200 ($q = 2.78$, $p < 0.05$) mg/kg.

3.2. *EPM test*

Figure 2 shows the percentage of time spent and entries in the open arm of the plus-maze. As expected, diazepam significantly increased percentage of entries ($q = 6.14$, $p < 0.001$) and time spent ($q = 7.04$, $p < 0.001$) in the open arms of the maze, demonstrating

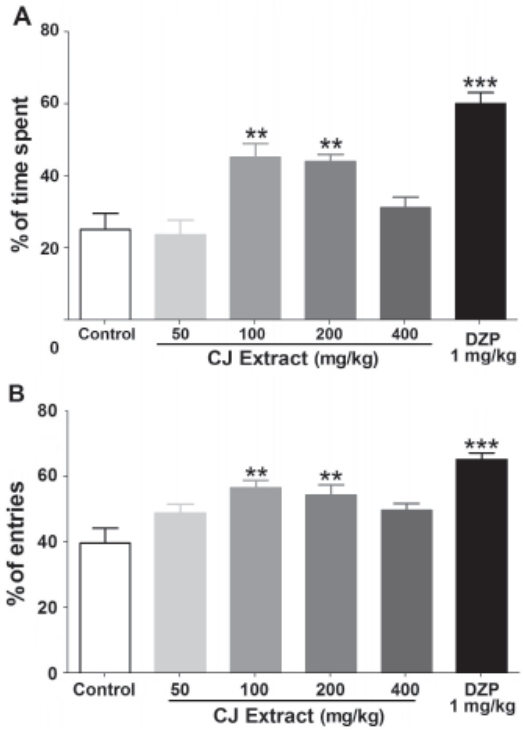

Figure 2. Effects of ethanol extract of CJ on elevated plus maze test in mice. Each bar represents the mean ± SEM (n = 8-10) of the percentage of time spent **(A)** or entries **(B)** into the open arm of the maze. ** $p < 0.01$, *** $p < 0.001$ significantly different from the control group. DZP, diazepam.

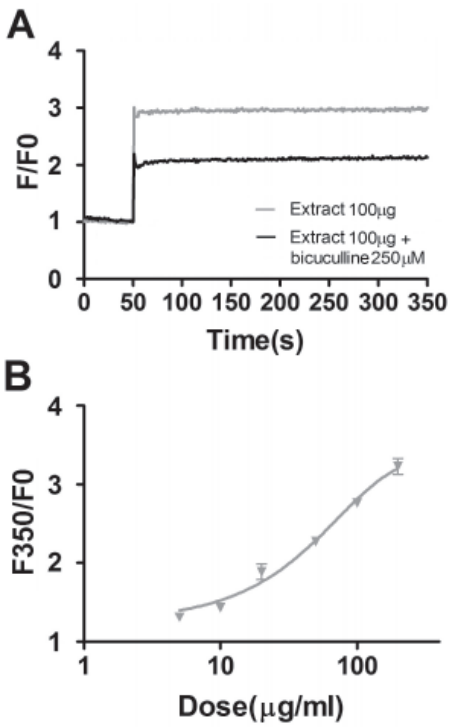

Figure 3. Effects of ethanol extract of CJ or CJ plus bicuculline on [Cl^-] influx in neuroblastoma cells. Fluorescence was monitored in the excitation wavelength at 365 nm and the emission wavelength at 450 nm using Cl^- sensitive indicator, MQAE. Contents of influx Cl^- were expressed as a peak (a.u.).

its anxiolytic effects (*18*). The CJ extract also showed profound increase in percentage of time spent [100 (q = 4.05, $p < 0.01$), 200 mg/kg (q = 3.82, $p < 0.01$)] and entries [100 (q = 4.06, $p < 0.01$), 200 mg/kg (q = 3.53, $p < 0.01$)], in the open arm of the plus maze.

3.3. *Intracellular Cl^- measurement assay*

Figure 3 shows the action of the CJ in cultured human neuroblastoma cell. Treatment of CJ extract increased Cl^- ion influx in human neuroblastoma cells, in a dose-dependent manner. Furthermore, this CJ-induced increment in the Cl^- influx was significantly blocked by the specific antagonist of the $GABA_A$ receptor, bicuculline (Figure 3).

4. Discussion

The present study shows that the ethanol extract of CJ produces anxiolytic-like effects in animals, as evaluated by the open-field test and the elevated plus-maze test. Furthermore, the CJ extract induced Cl^- ion influx in human neuroblastoma cells, suggesting that its anxiolytic effects are probably mediated through a GABAergic mechanism of action.

The open-field test is a simple way to measure general locomotor activity, willingness to explore as well as anxiety-like behaviors (*12*). In the present study, diazepam (1 mg/kg) caused significant decrease in exploratory activity, which is expected because of its known sedative properties (*16*). Treatment of the CJ extract did not profoundly alter general locomotion demonstrating that the CJ might not have, or might have very minimal, sedative properties. In addition, the animal's exploration in the center zone of the open-field was also evaluated. Activity in the center zone of the open-field can be a tool to evaluate the anxiolytic properties of a substance (*12*). Normally animals explore on the peripheral area and tend to remain close to the walls of the open-field, if a drug/substance has anxiolytic properties it would promote central zone exploration (*19*). Diazepam, a known anxiolytic drug, significantly increased central-zone exploration. This increase in center zone activity was also observed in animals treated with the CJ extract (100 and 200 mg/kg), reflecting a probable anxiolytic effects of CJ (Figure 1B). This anxiolytic-like activity of CJ extract, in the open-field test, was further supported by the findings in the EPM. The EPM test is the most widely used and accepted paradigm to measure anxiety-related behavior in rodents and considered to be a valid animal model of anxiety because it utilizes natural anxiogenic stimuli complementary to those observed in humans (*13,20*). Diazepam exhibited its established anxiolytic effects by significantly increasing the time spent and entries in the open arms of the maze. Increase in the proportion of time spent and entries into the open arms of the maze indicates reduction of anxiety (*17*). In a highly comparable manner, the CJ extract (100 and 200 mg/kg) significantly increased time spent and entries into the open arms of the maze. Taken altogether, our behavioral results suggest that the ethanol extract of CJ has anxiolytic properties comparable to that of diazepam, but with less sedative effects.

It can be observed from the results that the anxiolytic effects of CJ are not dose-dependent. Due to the limitations of the present study we cannot fully explain the exact reason behind this result, and that we can only infer based on previous reports/studies. Some studies on the anxiolytic effects of plant extracts, reported somewhat similar results (less effects on higher doses). This was attributed to the complex pharmacokinetic and pharmacodynamics of plant extracts (*21,22*). Plant extracts are composed of various components mostly with unknown pharmacological and dose-response data (*21*). It is possible that with higher dose complex interactions occur (probably between CJ and other bodily systems), which then altered the anxiolytic effects of the extract. Somewhat in support, is the *in vitro* finding (discussed below) wherein dose-dependent effects were observed when the CJ extract was administered directly to cultured human neuroblastoma cells, detouring some pharmacokinetic processes. It would be beneficial to investigate the individual effect of the extract in order to elucidate the

agents responsible for its anxiolytic effects. However, the present study is limited, thus further studies are needed to adequately address these issues. Nevertheless, the present results can be helpful for therapeutic decisions and with respect to toxicology.

It is thought that anxiety is a product of an imbalance in the brain; primarily between the excitatory (glutamate) and inhibitory (GABA) forces. In a normally functioning brain there is a delicate balance between excitatory and inhibitory forces, when this equilibrium is disrupted it would cause abnormalities in functioning (*23*). Wierońska *et al*. (*23*) asserted that this was the case in anxiety, the harmony between excitatory *versus* inhibitory forces in the brain is disrupted, such that GABA levels are decreased resulting to decrease inhibition leading to over-excitation. Indeed, this might be true because of the fact that GABA is the brain's principal modulatory (inhibitory) neurotransmitter, playing a very significant role in maintaining/regulating homeostatic milieu or equilibrium (*24*). Furthermore, supporting this hypothesis is the mechanism of action of one of the most commonly prescribed anxiolytic drug, the benzodiazepines (diazepam). Benzodiazepines alleviate anxiety by potentiating GABA neurotransmission, counterbalancing the over-excitation observed in anxiety (*25*). Specifically, it binds at the allosteric (benzodiazepine) site in the $GABA_A$ receptor resulting to a facilitation of the opening of its Cl^- sensitive ion channel (*24,26*). Based on all of these, we have decided to evaluate the effects of the CJ extract at the GABA receptor by measuring Cl^- influx in human neuroblastoma cells. Indeed, our results showed that the CJ extract facilitated Cl^- influx in a dose dependent manner (Figure 3). In addition, this increase in Cl^- influx was significantly blocked by co-administration of bicuculline, a specific and competitive antagonist of the $GABA_A$ receptor (*27*). These findings suggest that the anxiolytic-like effects of CJ might be mediated through a $GABA_A$ receptor-Cl^- ion channel mechanism of action.

In the present study we have observed a somewhat incongruent result in the sedative effects of diazepam and the CJ extract. Diazepam produced anxiolytic effects coupled with sedative effect. On the other hand, the CJ extract showed comparable anxiolytic effects but without profound sedative effects. Although, anxiolytic and sedative effects usually go hand in hand, there are reports which implied that anxiolytic and sedation are two dissociable things (*28*). In fact researchers have identified specific areas in the $GABA_A$ receptor wherein these effects are mediated. Sedation is mediated in the (α_1 and/or α_5) subunit of the GABA receptor, while anxiolytic is mediated in the (α_2 and/or α_3) subunit (*24*). Benzodiazepines (diazepam) targets all of these subunits, thus it manifest both sedative and anxiolytic effects (*29*). It is possible that the CJ extract acts on the subunits of the $GABA_A$ receptor which mediates anxiety, but not on the subunits which mediates sedation. This might probably explain the anxiolytic effects, void of significant sedative effects, manifested by the CJ extract. This particular effect may be beneficial, because the sedative effects associated with anxiolytic agents may sometimes be undesirable and even dangerous. Sedative drugs may cause deterioration of person's optimal daily functioning (*e.g.* work, school) or even predispose one to fatal harm (*e.g.* driving, falls, *etc*.) (*30*). This is the reason why scientists are on the search to find an anxioselective drug; and based on the present results CJ might be a promising candidate.

5. Conclusion

The results from present study provide evidences for the anxiolytic effect of the ethanol extract of CJ. In addition, CJ's anxiolytic effects are void of profound sedation suggesting that CJ is a promising anxioselective drug.

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Original Article

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New phenolic compounds from the twigs of *Artocarpus heterophyllus*

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ABSTRACT: Two new chalcones, artocarpusins A and B (1 and 2), one new flavone, artocarpusin C (3), one new 2-arylbenzofuran derivative, artocarstilene A (4), and 15 flavonoids were isolated from the twigs of *Artocarpus heterophyllus*. Their structures were established on the basis of extensive spectroscopic analysis. Compounds 9 and 16 showed moderate inhibitory activity on the proliferation of the PC-3 and H460 cell lines.

Keywords: *Artocarpus heterophyllus*, chalcone, flavone, 2-arylbenzofuran, cytotoxicity

1. Introduction

Artocarpus heterophyllus Lam, belonging to the family Moraceae (mulberry family) and popularly known as jackfruit, is a key tree found in home gardens in India and Bangladesh (*1-3*). Different parts of this species have been used for medicinal purposes, such as alleviating asthma and fever (the roots), relieving biliousness and diarrhea (the seeds), acting as a sedative for convulsions (the wood), stimulating lactation in women and animals and acting as an antisyphilitic and vermifuge in humans (the leaves), and relieving ulcers and wounds (the leaf ash) (*4,5*). The plant is known to produce prenylflavonoids, stilbenes, triterpenes, and sterols. Some of these compounds have exhibited interesting biological activities, such as cytotoxicity (*6*), antioxidative activity (*7*), anti-inflammatory activity (*8*), antimalarial activity (*9*), inhibition of tyrosinase and melanin biosynthesis (*10,11*), and inhibition of 5α-reductase (*12*). In the course of an ongoing search for anticancer metabolites from Chinese medicinal plants (*13*), the petroleum ether and EtOAc soluble fractions from the 90% EtOH extract of the twigs of *A. heterophyllus* were found to be active in an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) cell viability assay of the proliferation/survival of a panel of four cancer cell lines (PC3, H460, SF-268, and MCF-7). The crude extract was then investigated further and three new flavonoids, named artocarpusins A-C (**1-3**) (Figure 1), one new 2-arylbenzofuran derivative, named artocarstilbene A (**4**), and 15 known flavonoids (**5-19**) were obtained. Their structures were elucidated using spectroscopic methods, including 1D and 2D nuclear magnetic resonance (NMR) and high-resolution electrospray ionization mass spectrometry (HRESIMS). The cytotoxicity of these compounds to the PC-3 and H460 human cancer cell lines was tested.

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2. Materials and Methods

2.1. *General experimental procedures*

MTT was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Doxorubicin was purchased from Shenzhen Main Luck Pharmaceuticals, Inc. (Shenzhen, China). Infrared spectra (IR) were recorded on a Nicolet iN 10 Micro FTIR spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) in transmission mode. Ultraviolet spectra (UV) were obtained on a Shimadzu UV-2550 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). NMR spectra were measured on a Bruker Avance DRX-600 spectrometer (Bruker Biospin Group, Billerica, MA, USA) operating at 600 (^{1}H) and 150 (^{13}C) MHz with tetramethylsilane (TMS) as an internal standard. HRESIMS were carried out on a LTQ-Orbitrap XL (Thermo Fisher Scientific Inc., Waltham, MA, USA). All solvents used were of analytical grade (Laiyang Chemical Reagent Co., Ltd., Shandong, China). High-performance liquid chromatography (HPLC) was performed on an Agilent 1100 G1310A isopump equipped with an Agilent 1100 G1322A degasser, an Agilent 1100 G1314A VWD detector (210 nm) and a ZORBAX SB-C_{18} column (9.4 mm × 250 mm, 5 μm) (Agilent Technologies, Inc., Santa Clara, CA, USA). Silica gel (200-300 mesh;

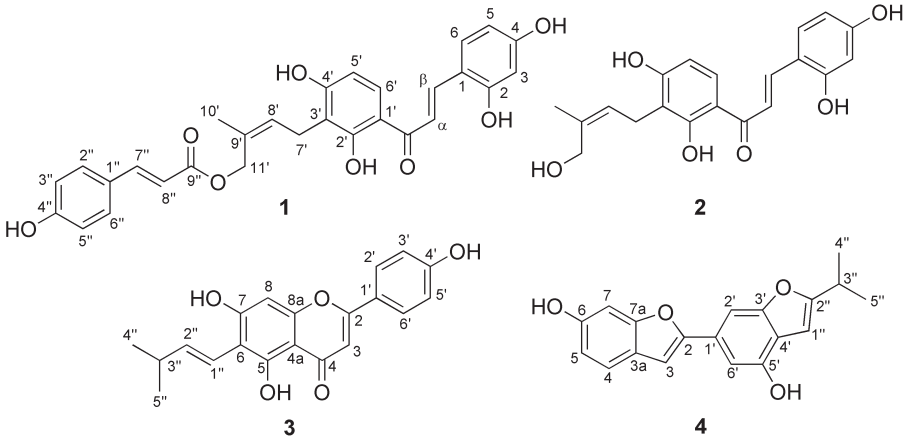

Figure 1. Structures of compounds 1-4.

Qingdao Haiyang Chemical Co., Ltd., Qingdao, China), C_{18} reversed-phase silica gel (YMC ODS-A gel, YMC Co., Ltd., Kyoto, Japan), MCI-gel (CHP20P, 75-150 μm, Mitsubishi Chemical Industries Ltd., Tokyo, Japan), and Sephadex LH-20 (GE Health, Uppsala Sweden) were used for column chromatography (CC). Thin layer chromatography (TLC) was carried out with high-performance TLC plates precoated with silica gel GF254 (Qingdao Haiyang Chemical Co., Ltd.). Spots of TLC were visualized within iodine vapor or by spraying with H_2SO_4-EtOH (1:9) followed by heating.

2.2. *Plant material*

The twigs of *A. heterophyllus* were collected from Xishuangbanna County, Yunnan Province, China in July 2009. The plant material was identified by Dr. Tao Shen, Shandong University. A voucher specimen (AH01-2009-07) was deposited with the Department of Natural Products Chemistry, School of Pharmaceutical Sciences, Shandong University.

2.3. *Extraction and isolation*

The air-dried and powdered plant material (10.0 kg) was extracted with 90% EtOH (4 × 20 L, each for 5 days) at room temperature. The combined extracts were concentrated under reduced pressure to yield a dark gum (150.0 g), which was suspended in H_2O, and fractionated successively with petroleum ether (5 × 1 L), EtOAc (5 × 1 L), and *n*-BuOH (5 × 1 L). The petroleum ether soluble fraction (10.0 g) had TLC like that of the EtOAc soluble fraction (50.0 g), hence they were combined together and subjected to silica gel CC eluted with petroleum ether-EtOAc (20:1→1:1) to yield 14 fractions (Fr. 1-Fr. 14). Fr. 6 (7.0 g) was subjected to CC of MCI-gel (MeOH-H_2O, 30:70→90:10) to yield six subfractions (Fr. 6.1-Fr. 6.6). Fr. 6.4 (1.1 g) was first subjected to CC of silica gel (petroleum ether-acetone, 20:1→2:1) to obtain the major portion, which was further separated by Sephadex LH-20 CC (MeOH) and then purified by preparative HPLC (MeOH-H_2O, 82:18, 1.8 mL/min) to yield **4** (5.9 mg, t_R = 9.06 min) and **7** (13.9 mg, t_R = 16.02 min). Fr. 6.6 (1.2 g) was first separated by CC of silica gel (petroleum ether-acetone, 20:1→1:1) to obtain the major portion, which was subjected to CC of C_{18} reversed-phase silica gel (MeOH-H_2O, 30:70→90:10) to yield **16** (8.0 mg). Fr. 7 (8.0 g) was subjected to CC of MCI-gel (MeOH-H_2O, 30:70→90:10) to yield six subfractions (Fr. 7.1−Fr. 7.6). Fr. 7.1 (0.72 g) was first subjected to Sephadex LH-20 CC (EtOH) and then purified by preparative HPLC (MeOH-H_2O, 78:22, 1.8 mL/min) to yield **18** (15.0 mg, t_R = 10.02 min). Fr. 7.3 (1.1 g) was first subjected to CC of silica gel (dichloromethane-acetone, 50:1→10:1) to obtain the major portion, which was further separated by CC of Sephadex LH-20 (MeOH) and then purified by preparative HPLC (MeOH-H_2O, 78:22, 1.8 mL/min) to yield **13** (8.0 mg, t_R = 16.02 min), **3** (10.5 mg, t_R = 19.50 min), **17** (5.0 mg, t_R = 22.02 min), and **15** (6.0 mg, t_R = 24.02 min). Fr. 8 (8.2 g) was further separated by a column of MCI-gel (MeOH-H_2O, 30:70→90:10) to yield seven subfractions (Fr. 8.1−Fr. 8.7). Fr. 8.3 (1.1 g) was first subjected to CC of silica gel (dichloromethane-acetone, 50:1→10:1) to obtain the major portion, which was further separated by Sephadex LH-20 CC (MeOH) and then purified by preparative HPLC (MeOH-H_2O, 75:15, 1.8 mL/min) to give **6** (5.1 mg, t_R = 20.06 min), **8** (10.1 mg, t_R= 21.26 min), and **14** (5.1 mg, t_R = 23.06 min). Fr. 9 (9.9 g) was subjected to CC of MCI-gel (MeOH-H_2O, 30:70→90:10) to yield eleven subfractions (Fr. 9.1−Fr. 9.11). Fr. 9.4 (1.2 g) was first separated by CC of silica gel (dichloromethane-acetone, 50:1→10:1) to obtain the major portion, which was purified by CC of C_{18} reversed-phase silica gel (MeOH-H_2O, 30:70→90:10) to yield **2** (8.0 mg) and **19** (20.0 mg). Fr. 9.5 (0.61 g) was first separated by Sephadex LH-20 CC (MeOH) and then purified by preparative HPLC (MeOH-H_2O, 78:22, 1.8 mL/min) to yield **5** (3.0 mg, t_R = 12.02 min). Fr. 9.9 (1.0 g) was first subjected to CC of silica gel (dichloromethane-acetone, 100:1→10:1) to obtain the major portion, which was further separated by Sephadex LH-20 CC (EtOH) and then purified by CC of silica gel (dichloromethane-

acetone, 50:1→10:1) to yield **9** (10.5 mg), **10** (7.5 mg), **11** (8.3 mg), **12** (3.3 mg), and **1** (2.5 mg).

2.4. *Cell lines and cell culture*

PC-3 cells (ATCC CRL-1435 human prostate adenocarcinoma) and NCI-H460 cells (ATCC HTB 177 human lung carcinoma) were cultured in RPMI-1640 medium (HyClone, Thermo Fisher Scientific Inc., Waltham, MA, USA). The medium was supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Carlsbad, CA, USA), 100 µg/mL penicillin, and 100 µg/mL streptomycin. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C.

2.5. *Cytotoxicity assay*

A tetrazolium-based colorimetric assay (MTT assay) was used to determine cell viability (*14*). The cancer cells were all cultured under standard culture conditions. The test compounds or vehicle control (dimethyl sulfoxide, DMSO) were added to appropriate wells and the cells were incubated for 72 h. Then MTT solution was added to the assay plates (final concentration, 0.5 mg/mL). After shaking for 10 sec, plates were returned to the incubator and incubated for 4 h. The supernatants were carefully removed and then100 µL of DMSO was added to each well to dissolve the precipitate. Next, the absorbance was measured at 570 nm with a Model 680 microplate reader (Bio-Rad, Hercules, CA, USA). The percent viability was expressed as absorbance in the presence of test compound as a percentage of that in the vehicle control. Doxorubicin and DMSO were used as positive and negative controls.

3. Results and Discussion

The petroleum ether and EtOAc soluble fraction was subjected to repeated column chromatography over silica gel, Sephadex LH-20, and HPLC to yield four new compounds **1**-**4**, together with 15 known ones, artocarmitin A (**5**) (*15*), 3'-[γ-hydroxymethyl-(*Z*)-γ-methylallyl]-2',4',4-trihydroxychalcone (**6**) (*16*), isobavachalcone (**7**) (*17*), 2',4',2,4-tetrahydroxy-3-(3-methyl-2-butenyl)-chalcone (**8**) (*18*), gemichalcones A (**9**) and B (**10**), isogemichalcone B (**11**) (*15*), artocarmitin B (**12**) (*15*), 6-(3-methylbut-2-enyl)-apigenin (**13**) (*19*), arthocarpesin (**14**) (*20*), norartocarpin (**15**) (*21*), artocarpin (**16**) (*22*), cudraflavone C (**17**) (*23*), 5,7,4'-trihydroxyflavone (**18**) (*24*), and norartocarpesin (**19**) (*25*). Compounds were identified by comparison of their spectroscopic data (*see Supplemental Data*) with values reported in the literature.

Artocarpusin A (**1**) was obtained as an orange powder. The molecular formula was established as $C_{29}H_{26}O_8$ according to HRESIMS with a pseudo molecular ion $[M + H]^+$ peak at *m/z* 503.1709 (calcd for $C_{29}H_{27}O_8$, 503.1706).

Table 1. ^{1}H- (600 MHz) and ^{13}C- (150 MHz) NMR data for compounds 1 and 2

Position	Compound **1**[a]		Compound **2**[b]	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1	---	115.2	---	114.1
2	---	160.0	---	159.3
3	6.53, d (2.0)	103.5	6.39, s	102.4
4	---	162.5	---	162.0
5	6.45, dd (8.5, 2.0)	109.1	6.32, d (8.5)	108.1
6	7.69, d (8.5)	131.7	7.72, d (8.5)	129.5
α	7.80, d (15.6)	117.4	7.68, d (15.5)	115.5
β	8.22, d (15.6)	140.9	8.07, d (15.5)	139.8
CO	---	193.4	---	192.0
1'	---	114.5	---	112.8
2'	---	165.0	---	161.7
3'	---	114.9	---	113.2
4'	---	162.3	---	163.4
5'	6.55, d (8.5)	107.9	6.46, d (8.8)	107.3
6'	7.92, d (8.5)	130.3	7.93, d (8.8)	130.5
7'	3.50, d (7.2)	22.1	3.27, d (7.2)	21.2
8'	5.58, t (7.2)	128.4	5.22, t (7.2)	123.4
9'	---	131.0	---	135.2
10'	1.75, s	63.5	1.65, s	20.8
11'	4.96, s	21.7	4.12, s	59.6
1"	---	127.0	---	---
2", 6"	7.57, d (8.5)	131.0	---	---
3", 5"	6.90, d (8.5)	116.6	---	---
4"	---	160.5	---	---
7"	7.63, d (16.0)	145.3	---	---
8"	6.39, d (16.0)	115.6	---	---
9"	---	167.6	---	---
2'-OH	14.26, s	---	14.18, s	---

[a] Data collected in acetone-d_6. [b] Data collected in DMSO-d_6.

The ^{1}H-NMR spectrum (Table 1) of **1** showed resonance for a methyl attached to a double bond at δ 1.75 (s, 3H), a methylene at δ 3.50 (d, *J* = 7.2 Hz, 2H), an oxygenated methylene at δ 4.96 (s, 2H), two *trans* olefinic protons at δ 7.63 (1H, d, *J* = 16.0 Hz, H-7") and 6.39 (1H, d, *J* = 16.0 Hz, H-8"), an olefinic proton at δ 5.58 (t, *J* = 7.2 Hz, H-8'), a 1,2,4-trisubstituted benzene ring at δ_H 6.53 (1H, d, *J* = 2.0 Hz, H-3), 6.45 (1H, dd, *J* = 2.0, 8.5 Hz, H-5), and 7.69 (1H, d, *J* = 8.5 Hz, H-6), a l,2,3,4-tetrasubstituted benzene ring at δ 6.55 (1H, d, *J* = 8.5 Hz, H-5') and 7.92 (1H, d, *J* = 8.5 Hz, H-6'), a *para*-disubstituted benzene ring at δ 6.90 (d, *J* = 8.5 Hz, H-3" and H-5") and 7.57 (d, *J* = 8.5 Hz, H-2" and H-6"), two olefinic protons of a chalcone skeleton at δ 7.80 (1H, d, *J* = 15.6 Hz, H-α) and 8.22 (1H, d, *J* = 15.6 Hz, H-β), and a hydrogen-bonded hydroxyl group at δ 14.26 (1H, s, 2'-OH). The ^{13}C-NMR spectrum (Table 1) revealed 29 carbon signals, which included the signals of a conjugated ketone carbonyl (δ 193.4 ppm), an esteric carbonyl (δ 167.6 ppm), three benzene rings, three double bonds, two methylenes (including one oxygenated at δ 63.5 ppm), and one methyl. The NMR data for **1** were very similar to those for 3'-[γ-hydroxymethyl-(*E*)-γ-methylallyl]-2,4,2',4'-tetra-hydroxychalcone 11'-*O*-coumarate (*26*), except for a change in the chemical shift values for H-8' and H-11'. The NOESY correlations of H-7'/H-11' and H-8'/H-10' (Figure 2) indicated that the double bond

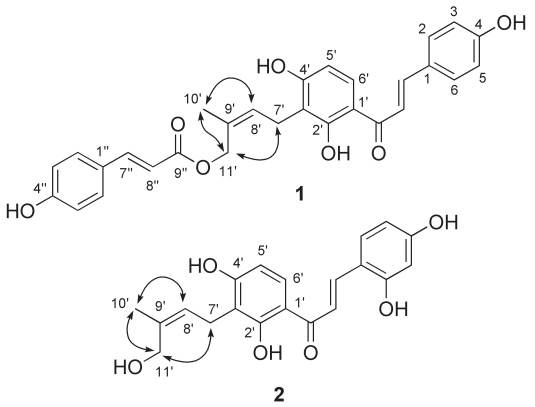

Figure 2. Key NOESY correlations (H↔H) of 1 and 2.

at C-8' was *Z*-configured. Thus, **1** was determined to be 3'-[γ-hydroxymethyl-(*Z*)-γ-methylallyl]-2,4,2',4'-tetrahydroxychalcone 11'-*O*-coumarate.

Artocarpusin B (**2**) was obtained as a yellow solid. HRESIMS revealed a pseudo molecular ion $[M + H]^+$ peak at *m/z* 357.1334 (calcd for $C_{20}H_{21}O_6$, 357.1338), which agreed with the molecular formula $C_{20}H_{20}O_6$. The ^{1}H- and ^{13}C-NMR data (Table 1) for **2** closely resembled those for **6** (*16*), except for the absence of signals for one of the methyls at C-9' and appearance of one oxygenated methylene (δ 4.12, s and δ 59.6 ppm, CH_2-11') in **2**. The NOESY spectrum showed intense correlations of H-10'/H-8' and H-11'/H-7', indicating that the double bond at C-8' was *Z*-configured. Thus, **2** was determined to be 3'-[γ-hydroxymethyl-(*Z*)-γ-methylallyl]-2',4',2,4-tetra-hydroxychalcone.

Artocarpusin C (**3**) was obtained as a yellow solid. HRESIMS revealed an $[M + H]^+$ ion peak at *m/z* 339.1227 (calcd for $C_{20}H_{19}O_5$, 339.1232), corresponding to the molecular formula $C_{20}H_{18}O_5$. The ^{1}H- and ^{13}C-NMR data (Table 2) for **3** were similar to those for isoartocarpesin (*18*) except for a difference in ring-B of the flavonoids. The absence of the AMX-type proton signals and presence of the AA'XX'-type proton signals at δ 7.90 (2H, d, *J* = 8.0 Hz, H-3' and H-5') and 6.91 (2H, d, *J* = 8.0 Hz, H-2' and H-6') indicated that **3** has a *para*-disubstituted phenyl moiety. Thus, **3** was determined to be 2'-dehydroxyisoartocarpesin.

Artocarstilbene A (**4**) was obtained as a brown solid. HRESIMS revealed an $[M-H]^-$ ion peak at *m/z* 307.0969, corresponding to the molecular formula $C_{19}H_{16}O_4$ (calcd for $C_{19}H_{15}O_4$, 307.0970). The ^{1}H- and ^{13}C-NMR data (Table 2) for **4** closely resembled those for moracin D (*27*), except for changing of the 2,2-dimethylpyran ring fused at C-3' and C-4' in moracin D to a 2-isopropylfuran ring at C-3' and C-4'. Further evidence for structure **4** came from HMBC correlations of H-1"/C-3', C-5', C-2", and C-3". Thus, **4** was determined to be 2'-isopropyl-2,6'-bibenzofuran-4',6-diol.

The flavonoids **2**, **3**, **5-10**, and **13-19** were evaluated for their *in vitro* inhibition of cell proliferation using

Table 2. ^{1}H- (600 MHz) and ^{13}C- (150 MHz) NMR data for compounds 3 and 4

Position	Compound **3**[a]		Position	Compound **4**[a]	
	δ_H (*J* in Hz)	δ_C		δ_H (*J* in Hz)	δ_C
2	---	163.5	2	---	154.7
3	6.73, s	103.0	3	7.17, s	101.2
4	---	182.0	3a	---	121.0
4a	---	103.0	4	7.37, d (8.0)	112.5
5	---	159.3	5	6.74, d (8.0)	121.0
6	---	108.7	6	---	156.3
7	---	163.5	7	6.94, s	97.9
8	6.48, s	94.2	7a	---	155.7
8a	---	155.7	1'	---	127.0
1'	---	121.6	2'	7.44, s	98.8
2'	7.90, d (8.0)	128.8	3'	---	151.1
3'	6.91, d (8.0)	116.4	4'	---	118.2
4'	---	161.6	5'	---	156.1
5'	6.91, d (8.0)	116.4	6'	7.08, s	104.0
6'	7.90, d (8.0)	128.8	1"	6.58, s	98.3
1"	6.52, d (16.2)	117.3	2"	---	163.5
2"	6.70, dd (16.2, 8.0)	140.4	3"	3.06, m	27.6
			4"	1.29, d (6.4)	20.8
3"	1.23, m	33.0	5"	1.29, d (6.4)	20.8
4"	1.05, d (6.2)	23.2			
5"	1.05, d (6.2)	23.2			
5-OH	13.88, s	---			

[a] Data collected in DMSO-d_6.

Table 3. Cytotoxicity of some compounds against human tumor cells

Compound	PC-3	H460
1	14.1 ± 0.3	16.2 ± 0.5
2	> 100	> 100
3	> 100	65.9 ± 7.1
4	> 100	> 100
5	41.6 ± 0.5	37.5 ± 4.4
6	26.2 ± 2.8	22.9 ± 1.0
7	15.6 ± 0.0	16.1 ± 1.6
8	22.2 ± 0.5	29.9 ± 0.5
9	8.2 ± 0.3	9.5 ± 0.4
10	9.8 ± 0.2	13.1 ± 1.6
11	14.9 ± 0.5	17.6 ± 1.1
12	11.2 ± 0.7	16.2 ± 0.5
13	43.8 ± 1.9	40.6 ± 1.7
14	16.3 ± 0.1	11.6 ± 0.3
15	22.3 ± 0.9	20.7 ± 0.7
16	7.9 ± 0.6	8.3 ± 0.4
17	16.0 ± 0.1	19.8 ± 1.9
18	75.4 ± 1.5	59.0 ± 1.1
19	47.7 ± 0.1	18.6 ± 3.0
Doxorubicin	0.2 ± 0.1	0.3 ± 0.1

Results are expressed as IC_{50} values in μM. Doxorubicin and DMSO were used as positive and negative controls.

two cancer cell lines, PC-3 (human prostate cancer cells) and H460 (human lung cancer cells). As shown in Table 3, compounds **9** and **16** showed cytotoxic activity with IC_{50} values of 8.2 ± 0.3 and 7.9 ± 0.6 μM for PC-3 and 9.5 ± 0.4 and 8.3 ± 0.4 μM for H460, respectively, and **10** showed inhibitory activity against PC-3 cells (IC_{50} = 9.8 ± 0.2 μM). Compounds **1**, **7**, **11**, **12**, and **14** showed weak activity against PC-3 and H460 cells (10 μM < IC_{50} < 20 μM). The other compounds showed

inactivity against PC-3 and H460 cells (IC_{50} > 20 μM). Doxorubicin was used as a positive control, with IC_{50} values of 0.2 ± 0.1 μM and 0.3 ± 0.1 μM against PC-3 cells and H460 cells, respectively.

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Original Article

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PAK1-deficiency/down-regulation reduces brood size, activates *HSP16.2* gene and extends lifespan in *Caenorhabditis elegans*

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ABSTRACT: There is an increasing evidence that the oncogenic kinase PAK1 is responsible not only for malignant transformation, but also for several other diseases such as inflammatory diseases (asthma and arthritis), infectious diseases including malaria, AIDS, and flu, as well as a series of neuronal diseases/ disorders (neurofibromatosis, tuberous sclerosis, Alzheimer's diseases, Huntington's disease, epilepsy, depression, learning deficit, *etc*.) which often cause premature death. Interestingly, a few natural PAK1-blockers such as curcumin, caffeic acid (CA) and rosmarinic acid (RA) extend the lifespan of the nematode *Caenorhabditis elegans* or fruit flies. Here, to explore the possibility that *C. elegans* could provide us with a quick and inexpensive *in vivo* screening system for a series of more potent but safe (non-toxic) PAK1-blocking therapeutics, we examined the effects of *PAK1*-deficiency or down-regulation on a few selected functions of this worm, including reproduction, expression of *HSP16.2* gene, and lifespan. In short, we found that PAK1 promotes reproduction, whereas it inactivates *HSP16.2* gene and shortens lifespan, as do PI-3 kinase (AGE-1), TOR, and insulin-like signalling /ILS (Daf-2) in this worm. These findings not only support the "trade-off" theory on reproduction *versus* lifespan, but also suggest the possibility that the reduced reproduction (or *HSP16.2* gene activation) of this worm could be used as the first indicator of extended lifespan for a quick *in vivo* screening for PAK1-blockers.

Keywords: PAK1, HSP16, GFP, CAPE, ARC, nematode, lifespan, RB689, trade-off, reproduction

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1. Introduction

Calorie restriction (CR) is well known to extend the lifespan of mice by activating the tumor-suppressing kinase (LKB1-AMPK) cascade which eventually activates the longevity and tumor suppressing transcription factor (FOXO) family by its phosphorylation and nuclear translocation (*1-3*). Interestingly, LKB1 inactivates an oncogenic kinase called PAK1, whereas it activates AMPK (*4*). In fruit flies (*Drosophila*) the gene "Methuselah" (*MTH*), encoding a G protein-coupled receptor (GPCR) of secretin receptor family, shortens the lifespan (*5,6*). Dysfunction of this gene (*6*) or the inhibition of MTH protein by its ligand antagonists (*7*) extends the lifespan by 35%. The MTH protein eventually activates the oncogenic RAS−PI-3 kinase cascade (*8*), leading to the activation of two oncogenic kinases, PAK1 and AKT. Thus, in both mammals and insects, PAK1 or AKT appears to be involved in the regulation of their lifespan somehow. In mammals both PAK1 and AKT are known to inactivate the longevity protein "FOXO" by the specific phosphorylation (*9,10*).

In the nematode *Caenorhabditis elegans* several distinct genes were identified as lifespan modulators. Among the positive regulators, are "*FOXO*" (*DAF-16*), "*HSP16.2*", and "*PTEN*" (*DAF-18*), whose dysfunction shortens the lifespan (*11-13*). Among the negative regulators, are insulin-like signalling (*ILS/DAF-2*), PI-3 kinase (*AGE-1*), *AKT*, and *TOR*, whose dysfunction extends the lifespan (*14-17*). If we understand correctly, these negative regulators shorten the lifespan mainly by inactivating the FOXO-HSP16.2 signalling pathway (*14-17*).

In this worm there are three distinct members of PAK family, PAK1, PAK2, and MAX-2 (*18-20*). Among them, the nematode PAK1 is most closely related to (and functionally same as) mammalian PAK1 which is essential for malignant growth of cells and several other diseases such as neurofibromatosis (NF) and Alzheimer's disease (AD) which often cause the premature death of human beings (*21,22*). So far no phenotype of *PAK1*-decient mutant of *C. elegans* (RB689) has been reported, except that the *PAK1* and *MAX-2* double mutant is

embryonically lethal, whereas *PAK1*-deficient mutant shows no apparent defect, suggesting that *PAK1* and *MAX-2* are functionally redundant in part (*19*). *MAX-2* is similar to *Drosophila PAK3*, whereas *PAK2* in this worm belongs to group II of PAK family (*PAK4-6*).

During our searching the potential phenotypes of RB689, or effects of PAK1-blockers, we found that RB689 shares a few common phenotypes with dysfunction of ILS, PI-3 kinase, and TOR in this worm and MTH in *Drosophila*: reduced brood size, increase in stress-resistance, and longer lifespan. Furthermore, we demonstrated the first example that *HSP16.2-GFP* (green florescent protein) fusion gene in this worm (CL2070) could be used as a potent visible indicator for a quick and inexpensive screening for "non-toxic" PAK1-blockers *in vivo* (combined with heat-shock).

2. Materials and Methods

2.1. *Strains of C. elegans and reagents*

Strains (RB689 and CL2070) of *C. elegans* were kindly provided by *C. elegans* Genomic Center (CGC). Caffeic acid phenethyl ester (CAPE) was purchased from Sigma Chemicals and artepillin C (ARC) was kindly provided by Dr. Hitoshi Hori of Tokushima University.

2.2. *Measurement of brood size*

The wild-type (N2) and RB689 of *C. elegans* were fed by the lawn culture of *Escherichia coli* (OP50) which was grown in the presence or absence of 200 μM CAPE or ARC on the standard nematode growth medium (NGM) agar plate for 2 days shortly after the hatching at 23°C. Then the number of eggs laid by each group of around 40 adult worms overnight (for 10 h) was counted. The brood size was calculated as the number of eggs per mother (female worm).

2.3. *Heat-shock–induced paralysis*

The wild-type (N2) and RB689 were fed by *E. coli* which was grown in the presence or absence of 200 μM CAPE or ARC for 2 days as described above. Then each group of 100 adult worms was heat-shocked at 35°C for 2 h. Then each group was cultured at 22°C for 4 h recovery, and then heated again at 35°C over 4 h. Every hour after the 2nd heat challenge, the number of worms in each group, which are "paralyzed" (not moved by a gentle touch with a platinum rod), was counted for scoring the survivers. The whole population of control wild-type group usually perished after 4 h, whereas more than a half of RB689 or CAPE/ARC-treated wild-type survived the 2nd heat-challenge.

2.4. *HSP16.2 dependent GFP expression*

The strain CL2070 which carries a transgenic reporter gene called "*HSP16.2-GFP*" fusion gene (*23*) was fed by *E. coli* (OP50) which was gown in the presence or absence of 200 μM CAPE overnight (12 h) at 22°C. Then each group of around 20 worms was heat-shocked at 35°C for 2 h, and then kept at 22°C for the recovery. After 4 h, each group was fixed with a drop (10 μL) of sodium azide (1 M) on slides for microscopy. Under blue light which stimulates the green florescence emission from GFP produced in each worm, the fluorescence images were acquired at the same exposure parameters, using a 40× objective of the microscope (BX60; Olympus, Tokyo, Japan) equipped with a digital camera (Micropublisher 5.0; QImaging, Burnaby, British Columbia, Canada).

2.5. *Measurement of lifespan*

The gravid hermaphrodites of the N2 and RB689 from the standard agar plates were washed, respectively. Then worms were dissolved in alkaline sodium hypochlorite in order to collect the eggs in utero. The released eggs were allowed to hatch by overnight incubation at 20°C in S-basal (*24*) to the age synchronous cultures of the L1 stage larvae. The lifespan of the hermaphrodites at 20°C was measured on the agar plates with the lawn culture of *E. coli* (OP50). In order to prevent progeny production, 5-fluoro-2'-deoxyuridine (FUdR; Wako Pure Chemical Industries Ltd., Osaka, Japan) was added to the agar plate at the final concentration of 40 μM after the animals had reached adulthood as described previously (*25*).

2.6. *Nuclear localization of FOXO (Daf-16)*

To detect the intracellular DAF-16 localization, pGP30 vector (*26*), in which the *Daf-16* gene transcript a2 (*daf-16*a2) is fused to GFP cDNA, was microinjected into each gonad of the N2 and RB689 animals at 100 ng/μL with pRF4 containing the *rol-6* (*su1006*) gene as described previously (*25*). The presence or absence of DAF-16 localization into the nucleus of the transgenic 4- and 10-day old animals was observed using a fluorescence microscope with digital imaging system (BX51TRF; Olympus Co., Tokyo, Japan).

3. Results

3.1. *PAK1-deficient strain (RB689) has smaller brood size than the wild-type (N2)*

When we started culturing the *PAK1*-deficient strain (RB689) on a plate for a few days, we noticed that eggs this strain laid are clearly fewer than those the wild-type produced, although all eggs are hatched and none of mutant adults shows any other detectable defect. Since the long-lived *C. elegans* strains, in which ILS, PI-3 kinase, or TOR dysfunctions, also show a significantly

smaller brood size (*14-16*), as does the longevity mutant (Methuselah) of *Drosophila* in which the *MTH* gene dysfunctions (*5,6*), we decided to measure the brood size of RB689, compared with those of the wild-type (N2) in the presence or absence of two distinct PAK1-blockers, CAPE and ARC from a bee product called propolis (*27,28*). As shown in Figure 1, the brood size of RB689 is only one 7th of that of N2 (non-treated wild-type strain), whereas both CAPE and ARC-treated wild-type populations produced basically same number of eggs as RB689, suggesting strongly that this phenotype is closely associated with reduced PAK1 activity in this worm. In other words PAK1 promotes the reproduction in this worm, as do RAS, PI-3 kinase, and FYN, the three upstream activators of PAK1 in this worm or mice (*14,29,30*).

3.2. *The PAK1-blocker CAPE activates HSP16.2 gene*

In mammals as well as yeasts, the oncogenic RAS-PI-3 kinase signalling cascade blocks a series of heat-shock genes, and as a consequence mammalian (or yeast) cells are rendered highly sensitive to heat shock treatment (*31*). Interestingly, two mammalian oncogenic kinases, PAK1 and AKT, phosphorylate distinct members of FOXO family, and inactivate their transcriptional function essential for the expression of heat-shock genes (*9,10*). In the nematode, FOXO is also essential for the transcription of heat-shock genes including *HSP16.2*, and the ILS-PI-3 kinase signalling cascade and TOR are known to inactivate the longevity (FOXO-HSP16.2) signalling cascade (*14-16*). However, it still remains to be clarified if AKT or PAK1 is involved in this PI-3 kinase mediated inactivation of FOXO-HSP16.2 signalling cascade. To monitor the possible role of PAK1 in the heat-sensitivity of this worm, we compared the heat-induced paralysis of this worm, between the wild-type and RB689 as well as the ARC/CAPE-treated wild-type. We observed that both RB689 and ARC/CAPE-treated worm are significantly resistant to the heat challenge than the non-treated wild-type population which perished in 4 h at 35°C (data not shown), suggesting that PAK1 appears to mediate the suppression of heat-shock genes in this worm as well.

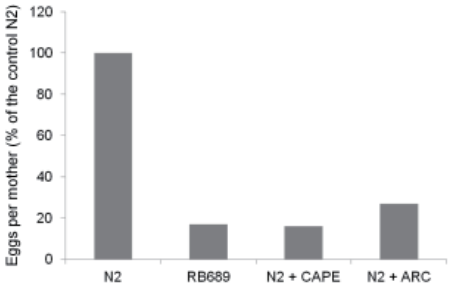

Figure 1. Brood size of RB689 compared with wild-type (N2) in the presence or absence of CAPE/ARC. Either *PAK1*-deficiency (RB689) or treatment of N2 with CAPE/ARC (200 μM) reduces the brood size (number of eggs/mother).

Thus, we examined the effect of CAPE, the PAK1-blocker which does not affect AKT, on the expression of *HSP16.2* gene, in an early stage of post heat-shock treatment of the strain CL2070, which carries a reporter gene, *i.e.,* the promoter of *HSP16.2* gene fused to *GFP* gene (cDNA). This fusion gene gradually produces a green florescent protein, only after heat shock, in this transparent worm over 24 h (*23*). If PAK1 suppresses the *HSP16.2* gene, CAPE (or any other "non-toxic" PAK1-blockers) could promote a rapid production of GFP shortly after the heat shock, and this florescent nematode system would provide us with a rapid and inexpensive *in vivo* screening for safe PAK1-blockers potentially useful for clinical application.

As shown in Figure 2, the strain CL2070 which has been treated with 200 μM CAPE overnight, started to glow rapidly even in 4 h after 2 h-heat shock, whereas the non-treated worm glowed only dimly in the same

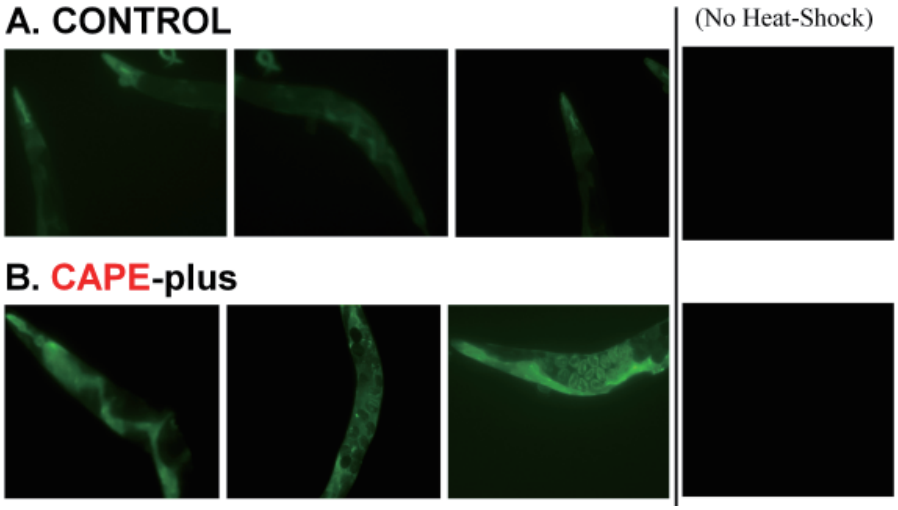

Figure 2. CAPE activates *HSP16.2* gene in *C. elegans*. 200 μM CAPE stimulates HSP16.2-GFP production in 4 h after the heat shock of *C. elegans* (CL2070). **(A)** The control CL2070; **(B)** CAPE-treated CL2070. Without heat shock, no GFP is produced.

period. The GFP expression occurred only after heat shock, and no GFP expression was detected without heat shock. In others words the *HSP16.2* gene promoter is regulated by at least two distinct factors. It is activated by heat shock, and inactivated by PAK1.

3.3. *RB689 lives longer than the wild-type*

According to numerous studies by Tom Johnson and his colleagues during last two decades or so, it was firmly established that the expression level of *HSP16.2* gene in this worm is well correlated to its lifespan (*32-34*). Thus, we compared the lifespan between RB689 (*PAK1*-deficient) and the wild-type under the standard conditions. As shown in Figure 3, RB689 lives significantly longer than the wild-type. Fifty percent of RB689 survived the first 27 days, whereas 50% of the wild-type survived only 17 days. In other words the *PAK1*-dificient worm could live 10 days (by around 60%) longer than the control worm, clearly indicating that PAK1 shortens lifespan of this worm, as do its upstream activators PI-3 kinase and ILS as well as TOR.

Does PAK1 inactivate FOXO in this worm, as it does in mammals? We think it does, based on our observation that nuclear localization of FOXO in RB689 is significantly increased in comparison with that in the wild-type, in particular at the early age (see Table 1). In addition, the PAK1-blocker CAPE activates *HSP16.2* gene expression, which depends on the transcription activity of nuclear FOXO.

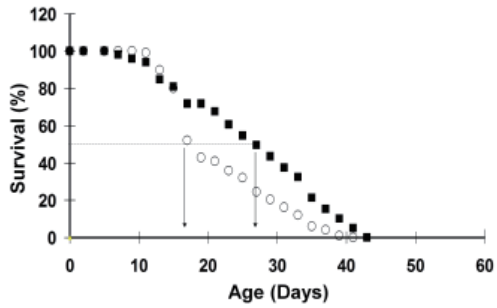

Figure 3. The effect of *PAK1*-deficiency on the lifespan. The survival rate between N2 (the control, circle) and RB689 (*PAK1*-deficient, square) strains of *C. elegans* was compared at 20°C on the standard agar plate. Fifty percent of RB689 survived on day 27, 10 days longer than the N2 counterparts.

Table 1. Effect of PAK1-deficiency on nuclear localization of FOXO

Strain	Age (days)	Nuclear FOXO/unit area
N2	4	1.6 ± 0.7
RB689	4	17.4 ± 1.6*
N2	10	11.0 ± 1.4
RB689	10	38.0 ± 7.4*

Numbers of GFP-stained nuclei were scored mainly in epithelia, muscles and intestine, and indicated as mean ± SD from more than three independent transgenic (FOXO-GFP)worms of each strain. * The numbers in RB689 are significantly larger than those in the control (N2) on both days 4 and 10 (by *t*-test, $p < 0.001$).

4. Discussion

Here we have shown three distinct phenotypes of *C. elegans* which lacks PAK1 or whose PAK1 is down-regulated by the PAK1-blocker CAPE. These phenotypes clearly indicate that PAK1 promotes the reproduction, inactivates *HSP16.2* gene and shortens the lifespan of this worm. These phenotypes are basically same as those of a few other "longevity" mutants of this worm, in which either ILS, PI-3 kinase, or TOR dysfunctions (*14-17*). Interestingly, these three phenotypes are also shared by the "longevity" mutant (Methuselah) of *Drosophila* in which *MTH* gene dysfunction (*5,6*). Since dysfunction of ILS, PI-3 kinase, and MTH eventually leads to the inactivation of PAK1, it is not a big surprise that these "longevity" mutants share the same phenotypes with RB689 or the CAPE-treated worm.

How about dysfunction of TOR? There is an evidence for a cross-talk between PAK1 pathway and TOR pathway (*35,36*). As shown in Figure 4, TOR requires another protein called Raptor for the full activation, and their interaction requires PAK1 (*35*). Conversely, TOR activates S6 kinase which in turn activates PAK1 (*36*). Thus, PAK1 and TOR form a vicious oncogenic cycle, and if TOR dysfunctions, PAK1 could be down-regulated accordingly.

In this context it should be worth noting that treatment of mice or fruit flies with rapamycin (Rapa), which directly inhibits TOR, also live longer than the control mice or fruit flies, respectively (*37,38*). To our great surprise, it was found recently that transgenic *RAC*-deficient fruit flies (*Drosophila*), in which the dominant negative (DN) mutant of RAC is expressed to block the oncogenic RAC-PAK1 signaling pathway, are extremely resistant to a variety of stresses such as heat, oxidants, desiccation, and starvation, and also can live for 40 days at 30°C, almost twice as long as the control flies which live for only 22 days (*39*).

Thus, a group at NIA in Baltimore recently started measuring the lifespan of PAK1-deficient (inflammation-resistant) mice (*40*) compared with that of controls. If PAK1 plays a similar role in the lifespan of mice, this study is expected to take around 5 years since the average lifespan of control mice is around 3 years. Nevertheless

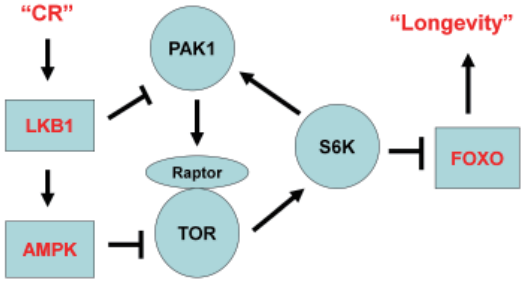

Figure 4. Cross-talk between PAK1 and TOR pathways. PAK1 and TOR form a vicious oncogenic cycle through Raptor and S6 kinase, respectively, to cause cancers in mammals and shorten lifespan of *C. elegans*.

it is most likely that *PAK1*-deficient mice live longer than controls, as do the Rapa-treated mice in which both TOR and PAK1 are down-regulated.

Interestingly, in 1999 Cynthia Kenyon's group at UCSF discovered that removal of germ-line cells by a laser micro-beam from *C. elegans* extends the lifespan by 60%, and this effect depends on the transcriptional function of FOXO (*41*). Along with this finding came an evolutionary "trade-off" theory noting that reduction in reproduction (fecundity) leads to extension of lifespan (*5,42*). In fact, over-expression of FOXO in adult *Drosophila* fat body caused an increased lifespan and reduced fecundity (*43*).

In other words, any compounds that reduce the brood size could extend the lifespan of *C. elegans* or *vice versa* (any "elixirs" could reduce the reproduction). However, curcumin was recently reported to extend lifespan of this worm, without affecting the reproduction, and even in the absence of FOXO (*44*). Curcumin is known to inhibit directly PAK1 as well as activates AMPK (*45,46*), and AMPK activates FOXO. Thus, it is likely that curcumin's life-extending property has nothing to do with its effect on either PAK1 or AMPK.

Nevertheless, we hypothesized that a PAK1-dependent "reproductive" signalling cascade from germ-line cells shortens the lifespan of this worm by inactivating FOXO. Then, based on our preliminary observation that ivermectin reduces the brood size of this worm by 90% at its sublethal dose (Grant W, *et al*., unpublished observation), we recently found that this old anti-parasitic drug also blocks PAK1 (*47*). Moreover, a natural PAK1-blocker called caffeic acid (CA) and its dimer called rosmarinic acid (RA) extend the lifespan of this worm (*48*). Thus, we are prompted to test this "trade-off" theory further by examining effects of several distinct PAK1-blockers such as CAPE, ARC, ivermectin, bitter anti-malaria drugs including berberine, glaucarubinone and "king of bitters" extract on *HSP16.2* gene expression and lifespan of this worm in no distant future. It is our "optimistic" hope that either reproduction assay or *GFP-HSP16.2* gene expression assay (or combination of these two), which can be done in a few days, may serve a quick *in vivo* screening for life-extending products (so-called "elixirs"), which would also be useful for therapy of cancers and several other PAK1-dependent diseases such as NF and AD without any side effect.

Lastly, it would be of great interest to test if *FYN*-deficient (or PP1-treated) mice live significantly longer than the control counterparts. Why? FYN is up-regulated by RAS, the major target of PP1, the SRC family-specific inhibitor, and contributes to the activation of PAK1 by 50% in RAS-transformed cells (*49*). Furthermore, the *FYN*-deficient mice are less fertile (up to 50%) compared with controls (*30*). Thus, if the "trade-off" theory is also applicable to mammals, *FYN*-deficient mice could be among the so-called "longevity" mouse family including "snell" dwarf mice in which the transcription factor Pit-1 dysfunctions, leading to growth hormone (GH) deficiency among others (*50*).

Interestingly, treatment of these dwarfs with GH and thyroxine increased their weight by approximately 45%, although they remained much smaller than controls. The hormone treatment also restored fertility to male dwarfs. However, this treatment did not diminish lifespan or lower their resistance to cataracts and kidney disease. Moreover, this dwarf is resistant to cancers and a variety of other diseases such as inflammation (*51*), as well as oxidative stresses such as 3-nitropropionic acid (3-NPA). In normal mice, 3-NPA activates MEK/ERK kinases and stimulates a robust phosphorylation of JNK at Ser63, whereas no phosphorylation takes place in snell mice, in response to 3-NPA (*52*). Since both MEK/ERK and JNK are the direct targets of PAK1, it is almost certain that in snell mice, PAK1 is somehow blocked or down-regulated. Again, these findings also strongly suggest, if not proven as yet, that *PAK1*-deficient (inflammation-resistant) mice could live significantly longer than controls, as does the *PAK1*-deficient nematode (RB689).

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Clinical benefits of concurrent capecitabine and cisplatin *versus* concurrent cisplatin and 5-flurouracil in locally advanced squamous cell head and neck cancer

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ABSTRACT: We aimed to assess the efficacy and safety of concurrent capecitabine and cisplatin over concurrent cisplatin and 5-flurouracil (5-FU) in locally advanced squamous cell carcinoma of the head and neck. One hundred and fifty-three patients (all of whom had stage III or IV unresectable disease with no distant metastases and who had received two cycles of taxol and cisplatin chemotherapy) were randomly assigned to receive either concurrent cisplatin (75 mg/m^2 in day 1 and 2) and 5-FU (750 mg/m^2 in day 1, 2, and 3) from the first day of radiotherapy at an interval of 3 weeks (Arm I) or cisplatin (75 mg/m^2 in day 1 and 2) and capecitabine (750 mg/m^2 in two divided doses from day 1-14) from the first day of radiotherapy at a 3-week interval (Arm II). Results showed that patients in Arm II had a significantly better rate of complete response, fewer nodes, and better overall response compared to those in Arm I. The two groups had a similar 3-year disease-free survival, progression-free survival, and overall survival, *i.e.* they did not differ significantly. Variables indicating the quality of life of the two groups were compared. Patients in Arm II had a significantly higher quality of life compared to those in Arm I. The two groups had similar treatment-related acute and late toxicity, *i.e.* they did not differ significantly. These results have thoroughly substantiated the contention that concurrent chemoradiation with capecitabine and cisplatin may be regarded as an effective and well-tolerated regimen in the treatment of the patients with locally advanced head and neck cancer.

Keywords: Head and neck cancer, capecitabine, chemoradiation

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1. Introduction

Most patients with squamous cell carcinoma of the head and neck present with loco-regionally advanced disease, which is associated with a poor prognosis despite all available treatment (*1*). Since the treatment has additional effects on functional abilities, such as speech and eating in these patients, recent attempts to improve the major end points of treatment (local control, organ preservation, and overall survival) have focused on the use of concurrent chemoradiation (*2-7*). Cisplatin combined with 5-flurouracil (5-FU) has been considered a standard regimen for concurrent chemoradiation (*3-5*). However, 5-FU has adverse effects such as oral mucositis, which is an additive complication of radiation, and myelosuppression; the adverse effects can result in treatment-related hospitalization or mortality, thereby diminishing quality of life and reducing compliance to treatment (*4,7*).

Therefore, a chemoradiotherapy regimen with reduced toxicity but maintained efficacy is needed. Oral fluoropyrimidine capecitabine was rationally designed to preferentially generate 5-FU in tumor tissue and mimic continuous-infusion 5-FU. This tumor selectivity is achieved by exploiting the significantly higher activity of thymidine phosphorylase (TP) in many tumor tissues compared to healthy tissue, thus reducing toxicity when used as a radiosensitizer (*8*). The expression of this enzyme is enhanced in tumor areas with poor perfusion, hypoxia, and acidosis; this situation is found in most advanced head and neck squamous cell carcinoma. There is evidence that radiation leads to the upregulation of thymidine phosphorylase expression, thereby synergizing the action of capecitabine (*9*). Mounting evidence suggests that oral capecitabine, which mimics continuous 5-FU infusion, has substantial activity in squamous cell carcinoma of the head and neck (*10,11*) and is replacing 5-FU in the treatment of many solid tumors as well as in advanced head and neck squamous cell carcinoma (*12-20*). Thus, the impact of radiation-induced toxicity on quality of life and treatment compliance justifies the use of concurrent chemoradiotherapy with an oral capecitabine-based regimen.

The objective of this study was to evaluate the benefits of concurrent capecitabine and cisplatin over concurrent cisplatin and 5-FU in locally advanced squamous cell head and neck cancer by assessing the response rate, survival, quality of life, and treatment-related toxicity during a three-year clinical follow up.

2. Materials and Methods

2.1. *Study protocol*

In total, 153 patients with head and neck cancer in a locally advanced stage (III, IV, M0) and a performance status of grade 1-2 seen by O.P.D from 2004-2005 were enrolled in the study. These patients were assessed thoroughly (history, clinical examination, and imaging studies) as shown in Table 1 and scheduled for treatment. All 153 patients were randomly divided to receive treatment in two arms. In Arm I, 75 patients received taxol (175 mg/m^2, day 1) and cisplatin (50 mg/m^2, day 2) administered as neoadjuvant chemotherapy in two cycles at a 21-day interval followed by concurrent chemoradiation where three cycles of cisplatin (75 mg/m^2 on days 1 and 2) and 5-FU (750 mg/m^2 on days 1, 2, and 3) at a 3-week interval along with radiotherapy at a dose of 70 Gy/35 fractions for 7 weeks. In Arm II, 78 patients received the same neoadjuvant chemotherapy regimen followed by concurrent chemoradiation with three cycles of cisplatin (75 mg/m^2 on day 1 and day 2) and capecitabine (750 mg/m^2 in two divided doses from days 1-14, with pyridoxine 200 mg in two divided doses on days 1-14) at a 3-week interval along with radiotherapy at a dose of 70 Gy/35 fractions for 7 weeks. Both arms are summarized in Table 2.

Table 1. Demographic and clinicopathological characteristics of two groups

Characteristics	Arm I (n = 67)	Arm II (n = 71)	p value
Age (years)			0.931
Mean ± SD	52.37 ± 11.10	52.54 ± 10.75	
Range	32-73	30-75	
Sex			0.380
Female	11 (16.4%)	8 (11.3%)	
Male	56 (83.6%)	63 (88.7%)	
Performance status			0.709
1	59 (88.1%)	61 (85.9%)	
2	8 (11.9%)	10 (14.1%)	
Site			0.573
Hypopharynx	14 (20.9%)	20 (28.2%)	
Larynx	22 (32.8%)	17 (23.9%)	
Oral cavity	11 (16.4%)	10 (14.1%)	
Oropharynx	20 (29.9%)	24 (33.8%)	
Histology:			0.457
Well differentiated	22 (32.8%)	17 (23.9%)	
Moderately differentiated	42 (62.7%)	49 (69.0%)	
Poorly differentiated	3 (4.5%)	5 (7.0%)	
Tumor:			0.492
T1	8 (11.9%)	4 (5.6%)	
T2	19 (28.4%)	22 (31.0%)	
T3	27 (40.3%)	34 (47.9%)	
T4	13 (19.4%)	11 (15.5%)	
Node:			0.766
N0	7 (10.4%)	6 (8.5%)	
N1	25 (37.3%)	30 (42.3%)	
N2	33 (49.3%)	31 (43.7%)	
N3	2 (3.0%)	4 (5.6%)	
Stage:			0.841
III	30 (44.8%)	33 (46.5%)	
IV	37 (55.2%)	38 (53.5%)	

Patients were evaluated 4-6 weeks after completion of treatment. The clinical benefits of concurrent chemoradiation with capecitabine and cisplatin in treating locally advanced squamous cell carcinoma of the head and neck were evaluated in terms of the response rate, 3-year overall survival and disease-free survival, radiation-induced toxicity, energy level (EL), activity level (AL), and overall quality of life (OQOL).

Informed written consent was obtained from each patient according to institutional regulations.

2.2. *Dose modification*

The protocol plan was continued despite mucositis or dermatitis. However, if grade 3 or 4 capecitabine/5-FU-related hematological or non-hematological toxicity such as mucositis, diarrhea, and hand-foot syndrome developed, capecitabine/5-FU was withheld until toxicity had improved by at least two grade levels. Subsequent capecitabine doses was reduced by 20%, doses of 5-FU was reduced by 20-25%. The dose of cisplatin was reduced to 50% if the calculated creatinine clearance level was 30-50 mL/min. No cisplatin was given if the creatinine clearance level was less than 30 mL/min. If myelosuppression (WBC count < 4,000/mm^3 or platelets count less than 100,000/mm^3) was present and a fever over 38°C or other clinically evident infection persisted, the cycle was postponed for 1 week or interrupted.

Table 2. Treatment given to two groups during the study

Treatment protocol	Arm I (n = 67)	Arm II (n = 71)
Neoadjuvant chemotherapy regimen	Taxol (175 mg/m^2, day 1), cisplatin (50 mg/m^2, day 2) × 3 weekly; 2 cycles	Taxol (175 mg/m^2, day 1), cisplatin (50 mg/m^2, day 2) × 3 weekly; 2 cycles
Concurrent chemotherapy	Cisplatin (75 mg/m^2, day 1 and 2), 5-FU (750 mg/m^2, day 1, 2, and 3) × 3 weekly; 3 cycles	Cisplatin (75 mg/m^2, day 1 and 2), capecitabine (750 mg/m^2, day 1-14) × 3 weekly; 3 cycles
	Radiotherapy, 70 Gy/35 fractions in 7 weeks	Radiotherapy, 70 Gy/35 fractions in 7 weeks

2.3. *Surgery*

Salvage surgery at the primary tumor site was recommended for eligible patients with residual disease who failed to achieve a complete response (CR) upon completion of treatment. Surgery in case of residual disease was carried out 6-8 weeks after completion of treatment.

2.4. *Study assessments*

Before starting treatment, all patients underwent a full medical history and physical examination, blood tests, computed tomography (CT) or magnetic resonance imaging (MRI) of the head and neck, and chest X-ray/CT-scan of the chest if lower neck nodes were involved.

Assessment of tumor response was done by clinical examination and imaging studies (X-rays, CT-scan) 4-6 weeks after completion of treatment. Biopsy or fine needle aspiration cytology was not routinely performed to determine pathological response; it was done only in the event of partial response/suspected lesions to confirm the presence of disease.

The definition of complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) was based on the standard definitions established by the World Health Organization (1979). Patients were monitored for acute chemoradiation-induced toxicity (by medical interview, physical examination, and complete blood count) weekly during chemoradiation; late radiation toxicity was assessed during follow-up. Toxicity was graded according to National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 3.0. Hand-foot syndrome was graded 1-3.

2.5. *Statistical analysis*

Continuous data were summarized as mean ± SD while discrete data (categorical) were indicated in %. Continuous variables were compared using an independent Student's *t* test while discrete variables were compared with a chi-square (χ^2) test. Groups were also compared by two-factor repeated measures analysis of variance (ANOVA) followed by a Bonferroni post hoc test after ascertaining normality and homogeneity of variance with a Shapiro-Wilk test and Levene's test, respectively. The survival of the two groups was compared using the Kaplan-Meier technique and the difference in the survival rate was determined with a Logrank test. A two-tailed ($\alpha = 2$) $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. *Basic characteristics*

The demographic (age, sex, and performance status) and clinicopathological (site, histology, tumor node and stage) characteristics of the two groups (Arm I and Arm II) at admission are summarized in Table 1. The age of patients in Arm I and Arm II ranged from 32-73 years and 30-75 years, respectively, with a mean ± SD of 52.37 ± 11.10 years and 52.54 ± 10.75 years, respectively. In both groups, patients were mostly male and most had a good performance status. In Arm I, the most prevalent site of the primary tumor was the larynx (32.8%); in Arm II, it was the oropharynx (33.8%). In both groups, most patients had tumors with a moderately differentiated (MD) histology; most tumors were class T3, lymph node involvement was N2, and tumors were stage IV. The two groups had similar basic characteristics, *i.e.* they did not differ significantly ($p > 0.05$).

3.2. *Treatment response*

Sixty-seven of the patients in Arm I completed the planned treatment while 71 in Arm II did so; the remaining 8 in Arm I and 7 in Arm II were lost to follow-up or patient refusal. The treatment response of the two groups was evaluated after completion of the respective treatments (4-6 weeks after completion of treatment) and after chemoradiation. Of the patients in Arm I, 36 were CRs (53.7%) and 28 were PRs (41.8%) while in Arm II 55 were CRs (77.5%) and 12 were PRs (16.9%). There were 38 (56.7%) primary and metastatic lymph node CRs and 36 PRs (53.7%) in Arm I while in Arm II there were 55 primary and metastatic lymph node CRs (77.5%) and 56 PRs (78.9%), as summarized in Table 3. Patients in Arm II had a significantly better CR, fewer nodes, and overall survival compared to those in Arm I. Furthermore, the dose reduction and treatment delay also lowered significantly in Arm II compared to those in Arm I.

3.3. *Survival*

After completion of 4-6 weeks of treatments, the two groups of patients were followed for three years. In both

Table 3. The treatment response of two groups

Characteristics	Arm I ($n = 67$)	Arm II ($n = 71$)	p value
Tumor			0.026
CR	38 (56.7%)	55 (77.5%)	
PR	26 (38.8%)	13 (18.3%)	
NR	3 (4.5%)	3 (4.2%)	
Node			0.005
CR	36 (53.7%)	56 (78.9%)	
PR	28 (41.8%)	12 (16.9%)	
NR	3 (4.5%)	3 (4.2%)	
Overall			0.006
CR	36 (53.7%)	55 (77.5%)	
PR	28 (41.8%)	12 (16.9%)	
NR	3 (4.5%)	4 (5.6%)	
Dose reduction	13 (19.4%)	5 (7.0%)	0.031
Treatment delay	16 (23.9%)	5 (7.0%)	0.006

groups, no death was observed during the treatments or end of the treatments. However, during follow up, 8 (10.6%) patients left the treatment and 13 (19.4%) died due to disease in Arm I while 7 (8.9%) and 9 (12.7%), respectively, in Arm II. Thus, at the end of the study, total 46 (68.6%) patients were found live in Arm I while 55 (77.4%) in Arm II. The 3-year disease-free survival (χ^2 = 0.89, p = 0.346), progression-free survival (χ^2 = 2.59, p = 0.107), and overall survival (χ^2 = 1.45, p = 0.229) of two groups were found similar *i.e.* did not differed significantly ($p > 0.05$) though it were 3.00 (HR = 3.00, 95% CI = 0.30-30.58)-, 2.34 (HR = 2.34, 95% CI = 0.83-6.41)-, and 1.67 (HR = 1.67, 95% CI = 0.72-3.89)-fold higher in Arm II compared to those in Arm I (Figure 1).

3.4. *Quality of life*

The changes in hemoglobin (Hb), weight, and quality of life were compared and summarized graphically (Figure 2) using 100 mm Linear Analog Scale Assessments

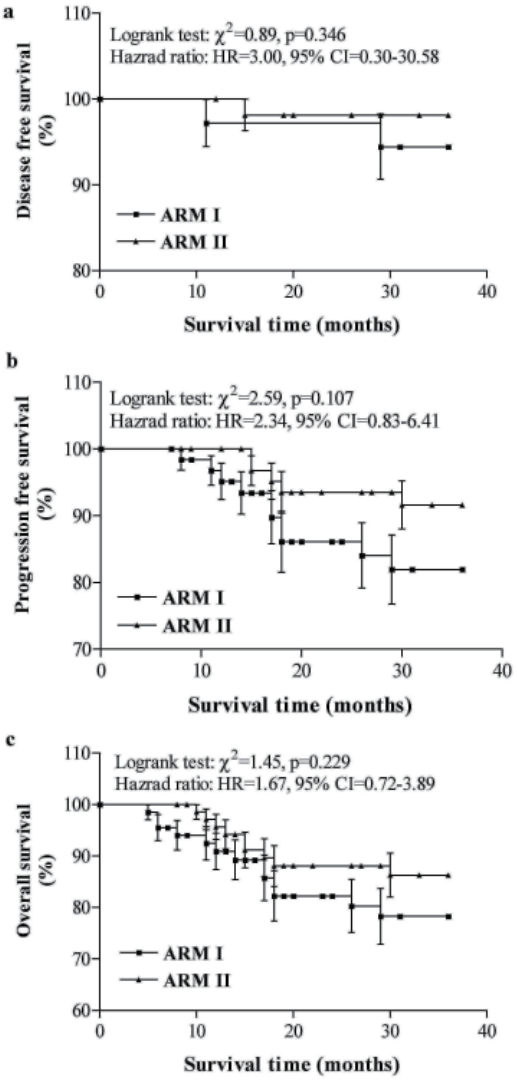

Figure 1. The 3-year disease-free survival (a), progression-free survival (b), and overall survival of two groups (c).

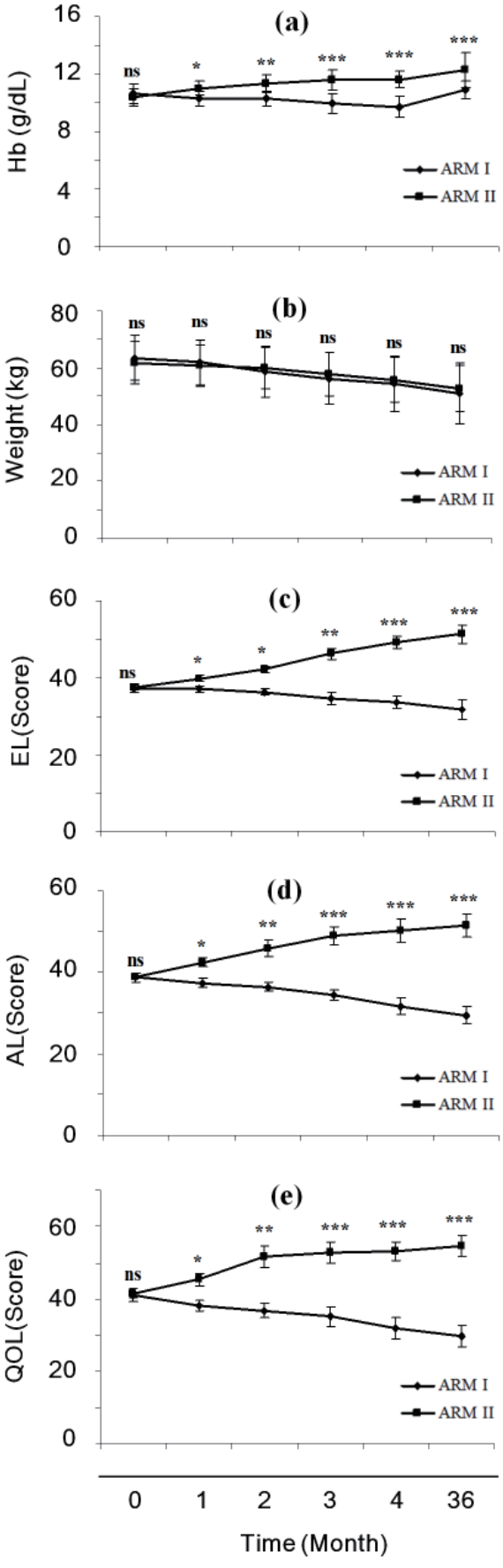

Figure 2. Improvement in Hb (a), weight (b), EL (c), AL (d), and QOL (e) for two groups. ns, $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

(LASAs) that rated the EL, AL, and OQOL of the two groups during the treatments and at end of the study. Results showed that patients in Arm II had significantly better results in terms of variables compared to those in Arm I except weight. A significant difference was evident after just 1 month of treatment and was more pronounced at the end.

3.5. *Toxicity*

At the end of the treatment, the treatment-related toxicity (hematological and non-hematological) in the two groups was also compared and is summarized in Table 4. The two groups had similar treatment-related toxicity, *i.e.* they did not differ significantly ($p > 0.05$).

The most severe hematologic adverse events were neutropenia and febrile neutropenia, which occurred with grade 3/4 intensity in 20 patients (29.8%) and 6 patients (9%), respectively, in Arm I while neutropenia and febrile neutropenia were observed in 26 patients (36.6%) and 18 patients (25.4%), respectively, in Arm II. These adverse events were successfully managed, and no treatment-related deaths occurred during this study. Mucositis and dermatitis, as would be expected from a combination of radiation with an effective chemotherapeutic-sensitizing agent, were the most common form of non-hematological toxicity. Grade 3/4 mucositis and dermatitis were observed in 70.2% of patients and 25.4% of patients, respectively, in Arm I while grade 3/4 mucositis and dermatitis were observed in 53.5% of patients and 33.8% of patients, respectively, in Arm II.

4. Discussion

Chemoradiation for head and neck cancer has led to significant improvements in survival, but these improvements have come at the cost of greater incidence of oropharyngeal mucositis and hematological toxicity, which are the most common reasons for unplanned treatment interruptions. Moreover, chemoradiation regimens to treat head and neck cancer are associated with troublesome adverse events that affect nutritional status and immune status, resulting in secondary infection, treatment interruption, and dose reduction that may compromise survival and quality of life. According to the literature, 80% of patients treated with chemoradiation have grade III or

Table 4. Treatment-related toxicity in two groups

Toxicity	Grade 1	Grade 2	Grade 3	Grade 4	Grade 3/4 (%)	*p* value
A: Hematological						
Anemia						0.323
Arm I	9 (13.4%)	13 (19.4%)	10 (14.9%)	14 (20.9%)	35.8	
Arm II	7 (9.9%)	8 (11.3%)	3 (4.2%)	3 (4.2%)	8.4	
Leukopenia						0.998
Arm I	37 (55.2%)	36 (53.7%)	21 (31.3%)	17 (25.4%)	56.7	
Arm II	26 (36.6%)	24 (33.8%)	14 (19.7%)	12 (16.9%)	36.6	
Neutropenia						0.371
Arm I	22 (32.8%)	14 (20.9%)	12 (17.9%)	8 (11.9%)	29.8	
Arm II	13 (18.3%)	11 (15.5%)	16 (22.5%)	10 (14.1%)	36.6	
Thrombo-cytopenia						0.224
Arm I	15 (22.4%)	17 (25.4%)	11 (16.4%)	11 (16.4%)	32.8	
Arm II	21 (29.6%)	9 (12.7%)	7 (9.9%)	13 (18.3%)	28.2	
Febrile neutropenia						0.147
Arm I	7 (10.4%)	5 (7.5%)	4 (6.0%)	2 (3.0%)	9.0	
Arm II	4 (5.6%)	10 (14.1%)	9 (12.7%)	9 (12.7%)	25.4	
B: Non-hematological						
Nausea						0.787
Arm I	33 (49.3%)	32 (47.8%)	7 (10.4%)	5 (7.5%)	17.9	
Arm II	39 (54.9%)	35 (49.3%)	10 (14.1%)	3 (4.2%)	18.3	
Vomiting						0.579
Arm I	31 (46.3%)	29 (43.3%)	4 (6.0%)	3 (4.5%)	10.5	
Arm II	25 (35.2%)	36 (50.7%)	6 (8.5%)	2 (2.8%)	11.3	
Renal dysfunction						0.660
Arm I	12 (17.9%)	7 (10.4%)	5 (7.5%)	3 (4.5%)	12.0	
Arm II	14 (19.7%)	5 (7.0%)	8 (11.3%)	6 (8.5%)	19.8	
Mucositis						0.365
Arm I	17 (25.4%)	33 (49.3%)	29 (43.3%)	18 (26.9%)	70.2	
Arm II	10 (14.1%)	29 (40.8%)	17 (23.9%)	21 (29.6%)	53.5	
Dermatitis						0.454
Arm I	36 (53.7%)	24 (35.8%)	11 (16.4%)	6 (9.0%)	25.4	
Arm II	31 (43.7%)	17 (23.9%)	15 (21.1%)	9 (12.7%)	33.8	
Diarrhea						0.873
Arm I	7 (10.4%)	6 (9.0%)	8 (11.9%)	5 (7.5%)	19.4	
Arm II	11 (15.5%)	13 (18.3%)	10 (14.1%)	7 (9.9%)	24.0	

IV hematological toxicity, 60% have grade III or IV mucositis, 20% have grade III or IV dermatitis, and 5% of patients die during concomitant chemoradiotherapy (*21*). Therefore newer chemotherapeutic agents that would cause less toxicity and have a high therapeutic index are needed for concurrent chemoradiation; however, no standard concurrent chemoradiotherapy regimen has been defined. Therefore, the present study was designed to evaluate the efficacy and toxicity of capecitabine instead of 5-FU in combination with cisplatin for concurrent chemoradiation to treat locally advanced squamous cell head and neck cancer.

In the current study, patients in Arm II had a significantly better CR, fewer nodes, and better overall response compared to those in Arm I, along with significant improvement in quality of life in terms of EL, AL, and OQOL. The incidence of mucositis, grade 3 or 4 neutropenia, and grade 3 or 4 febrile neutropenia differed only slightly in the two groups. However, the incidence of hematological toxicity in the current study differed significantly from that in previous studies using 5-FU-based regimens, where the incidence of grade 3 or 4 leukopenia was 29-81% (*1,18,22,23*).

Taxanes such as paclitaxel and docetaxel, which exhibit activity against squamous cell head and neck cancer, are increasingly being used in concurrent chemoradiation to improve treatment outcomes. These agents allow better locoregional control and result in better overall survival, but 34% of patients treated with the agents experienced grade 3 or 4 leukopenia and 3% patients died of treatment-related toxicity (*19*).

No hand-foot syndrome, a common complication associated with capecitabine, occurred in the patients in the current study, but the dose of capecitabine was reduced in two cycles in 5 patients due to neutropenia and mucositis and the dose of cisplatin was reduced in 13 patients because of nephrotoxicity and mucositis. The second and third cycle of chemotherapy was delayed in 16 patients (23.9%) in Arm I and 5 patients (7.0%) in Arm II due to mucositis and patient refusal to continue with the treatment.

The current study shows that although there is improvement in the response rate and quality of life in terms of EL, AL, and OQOL, there was no significant difference in survival and treatment-induced acute grade 3/4 toxicity profile. Improvement in quality of life for patients in Arm II may be attributed to a higher response rate among patients in this arm.

A number of prospective studies have also shown the efficacy and favorable safety profile of capecitabine as a radiosensitizer over other commonly used chemotherapeutic agents like cisplatin and 5-FU when treating many solid tumors, including head and neck cancer (*11-14,17,19-21,24*). The overall survival and progression-free survival rate in these studies at 2 years were 76.8% and 57.9%, respectively. Grade 3/4 mucositis and dermatitis were noted at rates of 67.6% and 24.3%, respectively. Grade 2 hand-foot syndrome, a complication of capecitabine, occurred in only 10.8% of patients. Encouraging results in terms of efficacy and the favorable safety profile of capecitabine have also been demonstrated in recent large phase-III studies comparing capecitabine with intravenous 5-FU and leucovorin to treat metastatic colorectal cancer (*18*).

Capecitabine has been widely used in the treatment of breast, stomach, and cervix cancer as well as other solid tumors (*11,13-16,19,20*). Several studies have demonstrated that concurrent chemoradiotherapy using capecitabine, with a dose ranging from 800 to 825 mg/m^2, in combination with cisplatin or oxaliplatin is effective and has a low toxicity profile in the neoadjuvant setting of rectal cancer or locally advanced esophageal cancer (*12-14*).

In conclusion, concurrent chemoradiotherapy with capecitabine and cisplatin was found to be well tolerated and effective in patients with locally advanced head and neck cancer. Accordingly, this regimen can be considered beneficial and may form an important therapeutic treatment option for locally advanced head and neck cancer, meeting the needs and assuaging the concerns of patients. However, further investigations in prospective studies with larger samples and a longer follow up are required to evaluate this regimen.

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Case Report

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Efficacy of ranolazine in a patient with idiopathic dilated cardiomyopathy and electrical storm

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ABSTRACT: A case of idiopathic dilated cardiomyopathy with an arrhythmic storm refractory to the usual antiarrhythmic therapy will be reported. The idiopathic structural heart disease of the patient is a vulnerable anatomic substrate in itself, for electrical instability and reentry mechanism, because of heterogeneous areas of scarred myocardium and low left ventricle ejection fraction. In this case, the ranolazine administration was safe and effective for the prevention of further electrical storms.

Keywords: Idiopathic dilated cardiomyopathy, electrical storm, anti-arrhythmic drugs refractoriness, ranolazine

1. Introduction

The term electrical storm (ES) describes a state of electrical instability of the heart characterized by a series of malignant ventricular arrhythmias in a short period of time (*1*). The most accepted definition is "Three or more distinct episodes of ventricular tachycardia (VT) or ventricular fibrillation (VF) in 24 h, requiring the intervention of the defibrillator (anti-tachycardia pacing (ATP) or shock)" (*1,2*). According to the above-mentioned definition, the reported incidence of ES changes according to the studied populations: the incidence ranges from 10% to 28%, in those studies in which implantable cardioverter defibrillator (ICD) implantation is carried out for secondary prevention (*1*), but it is substantially lower − about 4% in the MADIT II study (*3*), which concerned primary prevention.

Electrical storm is, therefore, an increasingly common and life-threatening syndrome (*2*): the rapid succession of ventricular arrhythmias and multiple ICD shocks lead to increased mortality immediately after the event (*1*). Both arrhythmic events and repeated shocks can cause inflammation, remodelling, myocardial damage, left ventricular systolic dysfunction, progression of heart failure (*2*), and even anxiety, depression, and post-traumatic stress syndrome (*2*). Electrical storm is a very challenging clinical event and it is difficult to manage and to prevent.

At the moment amiodarone, sotalol, and β-blockers are the mainstay of therapy for the prevention of ventricular arrhythmias and electrical storm (*2,4*). Although they are usually effective, some patients are drug refractory, that means they have recurrent episodes of myocardial electrical instability, despite amiodarone and β-blockers combined (*2*). In addition, long-term amiodarone therapy is responsible for substantial side-effects (pulmonary fibrosis, hypothyroidism, liver toxicity, and corneal deposits) (*2*). Unfortunately no other antiarrhythmic drugs are currently available and efficacious to reduce the incidence of an electrical storm.

2. Case report

We describe the case of a 75 years old man, affected by idiopathic dilated cardiomyopathy and severe left ventricular systolic dysfunction (LVEF 30%), who received an ICD implantation for primary prevention in March 2010. The patient regularly carries out periodic outpatient controls at our Electrophysiology Center, to assess any tachi-arrhythmic episodes and the functional status of the device. In addition a remote monitoring system follows the device day by day.

From November 2011 to January 2012, an electrical storm and recurrent episodes of monomorphic VT have been reported (Figure 1). In particular, the electrical storm consisted of two subsequent fast monomorphic VT beating at a heart rate of 220 beats per minute (bpm), each sustained for 1 min and 40 sec and interrupted by two ICD interventions, followed by a slower VT (170

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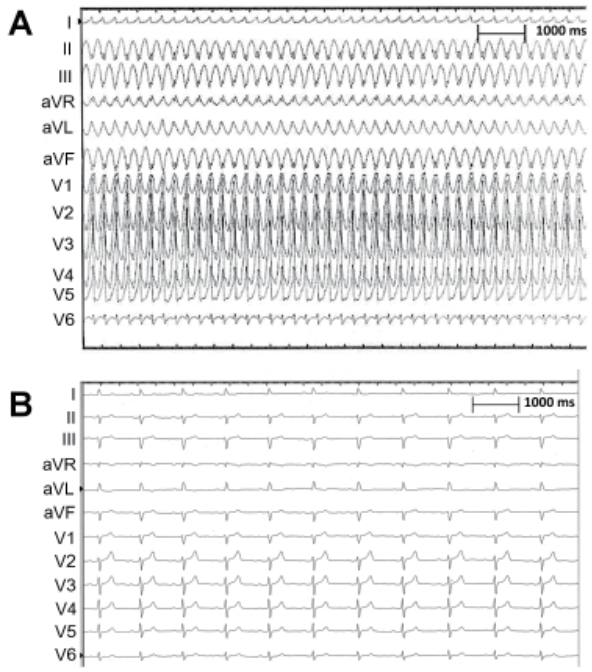

Figure 1. Twelve lead-electrocardiogram. (A) Monomorphic fast ventricular tachicardia (hear rate: 220 bpm); **(B)** Restoration of sinus rhythm after ICD-DC shock at 41 Joule.

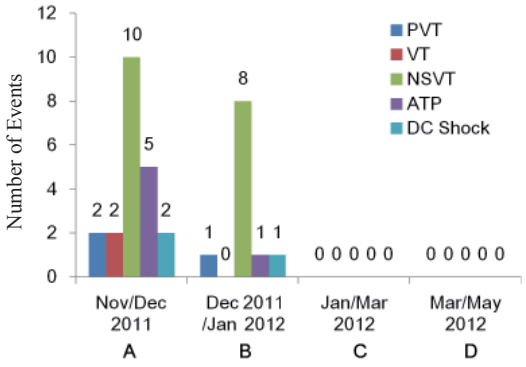

Figure 2. Diagram illustrating the relation between the ventricular events and drugs over time. Treatment regimens: **(A)** carvedilol 50 mg, amiodarone 200 mg, and ramipril 5 mg per day; **(B)** bisoprolol 10 mg, amiodarone 200 mg, flecainide 100 mg, and ramipril 5 mg per day; **(C)** bisoprolol 10 mg, amiodarone 200 mg, ranolazine 375 mg × 2, and ramipril 5 mg per day; **(D)** bisoprolol 10 mg, amiodarone 200 mg, ranolazine 375 mg × 2, and ramipril 5 mg per day. FVT, fast ventricular tachycardia; VT, ventricular tachycardia; NSVT, non-sustained ventricular tachycardia; ATP, anti-tachycardia pacing; DC shock, direct current shock.

bpm), ended by means of an ATP run. In that occasion the patients fainted, he was brought to the emergency room and then admitted to the cardiac Intensive Care Unit.

The most common triggers and risk factors of ventricular arrhythmias (acute myocardial infarction, worsening heart failure, electrolyte imbalance, chronic renal failure, hyperthyroidism, infections and fever, inherited arrhythmic syndromes) (*2*) were promptly excluded. Intracardiac electrocardiograms, that were recorded during the episodes, demonstrated that a premature ventricular complex, in the presence of an established heart disease, was the trigger.

Consequently, it was immediately decided to maximize the heart failure therapy, for the prevention of consequent ventricular arrhythmias. So, the patient was first treated with carvedilol 50 mg/die, amiodarone 200 mg/die 7 days/week, ramipril 5 mg/die, furosemide, and canrenoate potassium. Flecainide was added (100 mg/die) too, because of a single episode of atrial fibrillation with very high ventricular response, then degenerated in VF. Soon after, carvedilol was replaced by bisoprolol 10 mg/die and flecainide was suspended because of inefficacy. Despite the maximized antiarrhythmic therapy, the patient was drug refractory and still suffered from ventricular tachycardias' episodes. Therefore, considering the optimized heart failure therapy and the patient's clinical stability (NYHA I-II), ranolazine (375 mg tablets, twice daily) was introduced into the therapeutic scheme: it had been administered off label, as an antiarrhythmic agent (Figure 2). From January 2012 to May 2012, only few ventricular extrasystoles have been reported (neither ICD interventions nor FVT/VF) and the patient refers to feel well.

3. Discussion

Electrical storm is an increasingly common and life-threatening syndrome and a very challenging clinical event and it is difficult to manage and to prevent. Unfortunately, few antiarrhythmic drugs are currently available.

Ranolazine is an antianginal drug that inhibits a number of ion currents that are important in the genesis of the transmembrane cardiac action potential. It was initially developed as an antianginal agent, but it was found to additionally exert antiarrhythmic actions (*5*).

In the ventricles, ranolazine inhibits the late phase of inward sodium current (late INa), an expected effect to shorten action potential duration, and it reduces the rapidly activating delayed rectifier potassium current (IKr), an expected effect to lengthen action potential duration. In atria, in addition to blocking late INa and IKr, ranolazine inhibits the early or peak sodium channel current (peak INa) (*6*). It also has some effects on other currents, such as ICaL, INa-Ca, IKs. Based on its effects on these channels, some authors have proposed ranolazine as an antiarrhythmic agent (*5*).

In the ventricles, ranolazine effectively suppresses arrhythmogenesis associated with the reduced depolarization reserve caused by an increased late Ina, reduced IKr, or a combination of both (*6*). The group of Antzelevich *et al*. first pointed out the effect of ranolazine to suppress early after depolarizations (EADs) and delayed after depolarizations (DADs) (*6*). These observations first suggested an alleged role of ranolazine in preventing arrhythmias. In isolated perfused hearts of guinea pig and rabbit, ranolazine

has been shown to suppress EADs and VT induced by drugs that block IKr (*7*). Dhalla *et al*. observed that ranolazine reduced ventricular arrhythmias (such as ventricular premature beats, VT and VF) induced by ischemia and ischemia/reperfusion in an anesthetized rat model with transient (5 min) ligation of the left coronary artery, followed by reperfusion: in particular, ranolazine significantly reduced the incidence of ventricular fibrillation (67% in controls *vs*. 42% ($p = 0.414$), 30% ($p = 0.198$), and 8% ($p = 0.0094$) in ranolazine at 2, 4, and 8 μM respectively) (*8*). Similar results were obtained recently by Kloner *et al*. (*9*): they have for the first time compared ranolazine with other antiarrhythmic agents, like sotalol and lidocaine, as therapeutic doses, in an ischemia/reperfusion model. They observed that ranolazine was effective as much as sotalol or lidocaine in reducing reperfusion-induced ventricular arrhythmias: in fact, the incidence of ventricular arrhythmias in the sotalol, lidocaine, ranolazine, and control groups was 7/20, 10/20, 9/20, and 16/20, respectively.

In conclusion we describe a case of idiopathic dilated cardiomyopathy with an arrhythmic storm refractory to the usual antiarrhythmic therapy. Ranolazine, in association with carvedilol and amiodarone, suppresses arrhythmogenesis in this patient.

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