Withanolides-Induced Breast Cancer Cell Death Is Correlated with Their Ability to Inhibit Heat Protein 90

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Abstract

Withanolides are a large group of steroidal lactones found in Solanaceae plants that exhibit potential anticancer activities. We have previously demonstrated that a withanolide, tubocapsenolide A, induced cycle arrest and apoptosis in human breast cancer cells, which was associated with the inhibition of heat shock protein 90 (Hsp90). To investigate whether other withanolides are also capable of inhibiting Hsp90 and to analyze the structure-activity relationships, nine withanolides with different structural properties were tested in human breast cancer cells MDA-MB-231 and MCF-7 in the present study. Our data show that the 2,3-unsaturated double bond-containing withanolides inhibited Hsp90 function, as evidenced by selective depletion of Hsp90 client proteins and induction of Hsp70. The inhibitory effect of the withanolides on Hsp90 chaperone activity was further confirmed using in vivo heat shock luciferase activity recovery assays. Importantly, Hsp90 inhibition by the withanolides was correlated with their ability to induce cancer cell death. In addition, the withanolides reduced constitutive NF-κB activation by depleting IκB kinase complex (IKK) through inhibition of Hsp90. In estrogen receptor (ER)-positive MCF-7 cells, the withanolides also reduced the expression of ER, and this may be partly due to Hsp90 inhibition. Taken together, our results suggest that Hsp90 inhibition is a general feature of cytotoxic withanolides and plays an important role in their anticancer activity.

Introduction

Withanolides are a large group of steroidal lactones found in Solanaceae plants that exhibit potential anticancer activities. Some withanolide-containing plants have been known as folk remedies for centuries. For example, Withania somnifera contains a major withanolide- withaferin A, has been traditionally used in India for treatment of arthritis and other musculoskeletal conditions, and as a general tonic [1]. Pharmacological studies have revealed that besides anti-inflammatory activity, withanolides possess immunoregulatory, anti-tumor, anti-angiogenic, anti-invasive, and chemopreventive effects [2]. In addition, there are over 350 withanolides isolated and identified to date [3]; the diverse structural analogs provide a great opportunity for the study of structure-activity relationships, target identification, and lead optimization. Therefore, withanolides represent promising lead compounds for development of new anticancer drugs.

In our previous study, we have found that tubocapsenolide A, a novel withanolide from a native Taiwanese plant Tubocapsicum anomalous (Franchet and Savatier) Makino (Solanaceae), exhibited significant cytotoxicity against a panel of cancer cell lines [4]. Further study on the mechanism of action revealed that tubocapsenolide A inhibited the function of heat shock protein 90 (Hsp90) through thiol oxidation, and thus caused the depletion of Hsp90 client proteins, including Akt, CDK4, cyclin D, and mutant P53 [5]. Because Hsp90 client proteins play critical roles in the regulation of cell proliferation, survival, and apoptosis, it is suggested that tubocapsenolide A exerted its anticancer effect by inhibiting Hsp90. Subsequent to our study, Yu et al. reported that another withanolide withaferin A also has the same inhibitory effect on Hsp90 [6]. More recently, by using molecular docking approach, Grover et al. predicted that WA has the potential to inhibit the association of Hsp90 to its co-chaperone Cdc37 by disrupting the stability of attachment of Hsp90 to Cdc37 [7]. Therefore, we wondered whether the Hsp90-inhibitory effect is a general characteristic of withanolides and contributes to their anticancer effect. In the present study, we compared nine withanolides with different structural features for their cytotoxicity and Hsp90-inhibitory activity, and found a good correlation between these two effects. In addition, structure activity analysis revealed a critical requirement of the β, β-unsaturated ketone unit in the ring A of withanolides for the inhibitory effect on Hsp90.

Materials and Methods

Materials

Tubocapsenolide A, tubocapsenolide B, tubocapsanolide C, tubocapsanolide E, and anomanolide A were isolated from...
Tubocapsicum anomalum as described previously [4]. 4β-Hydroxy-withanolide, withanolide E, and peruvianolide H were isolated from Physalis peruviana [8]. Withaferin A was purchased from ChromaDex (Irvine, CA). The purities of the withanolides used in the study are over 98% determined by high pressure liquid chromatography. The withanolides were dissolved in DMSO, and the final concentration of DMSO in culture medium was 0.1%. Anti-Poly(ADP-ribose)polymerase, anti-caspase-3, anti-Raf-1, anti-CDK4 antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-Akt, anti-IKKα, anti-IKKβ, anti-IkB, anti-phospho-IkB, anti-NF-κB, and anti-phospho-NF-κB antibodies were from Cell Signaling Technology (Beverly, MA). Anti-Hsp90 and anti-Hsp70 antibodies were from Stressgen Biotechnologies (San Diego, CA). ONE-Glo™ luciferase assay system was purchased from Promega (Madison, WI). Geldanamycin, N-acetylcysteine, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and all other chemicals were obtained from Sigma Chemical Co (St Louis, MO).

Cell culture

MDA-MB-231 and MCF-7 human breast cancer cells were obtained from American Tissue Culture Collection. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2 in RPMI 1640 medium, supplemented with 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml). The cells were harvested by trypsinization and plated 24 h before treatment with the test drugs.

Evaluation of cell viability

1×10⁴ cells were plated into each well of a 96-well plate and treated with the various concentrations of drugs for different indicated times. At the end of each time point, 100 μl of MTT solution (0.5 mg/ml) was added to each well. Cells were then incubated at 37°C for 1 h. The MTT crystals in each well were solubilized with 100 μl of DMSO. Absorbance was read at 550 nm.

Western blot assay

Following exposure to various drugs, cancer cells were washed with PBS and lysed for 10 min at 4°C in a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 20 μg/ml leupeptin, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 5 mM sodium fluoride, and 5,000 units/ml aprotinin). Equal proteins were separated by SDS-
PAGE and transferred to a nitrocellulose membrane, followed by visualization using the enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech). For analysis of disulfide-linked Hsp90 by non-reducing SDS-PAGE, drug-treated cells were washed with PBS at the indicated periods and then incubated with iodoacetamide (40 mM) for 5 min to prevent thiol-disulfide exchange and post-lysis oxidation of free cysteines [9]. Moreover, samples were diluted in SDS-sample buffer without reducing agents before loading onto SDS-polyacrylamide gels.

Annexin V/propridium iodide (PI) staining

Annexin V/PI staining was performed as previously described [10]. Cancer cells (2×10^5 cells) were seeded onto each well of a 6-well plate and treated with DMSO or test drugs for 48 h. Treated cells were harvested by trypsinization and stained with annexin V and PI for 15 min at room temperature in the dark according to the manufacturer’s instructions (BD Pharmingen). Apoptotic cells were quantified by flow cytometry (Beckman Coulter, EPICS XL-MCL). Stained cell populations were defined as: lower left quadrant, living cells (annexin V^-/PI^-); lower right quadrant, early apoptotic cells (annexin V^-/PI^+); upper right quadrant, late apoptotic cells (annexin V^+/PI^+); upper left quadrant, primary necrotic cells (annexin V^-/PI^+).

In vivo Hsp90 chaperone activity assay

The measurement of Hsp90 chaperone activity in intact cells was carried out by the methods of Schneider et al. [11] and Hernández et al. [12] with slight modifications. MDA-MB-231 cells were transfected with a pGL2-sv40-luciferase plasmid. The luciferase-expressed cells were treated with DMSO or test drugs at 37°C for 30 min, and then transferred to 42°C for another 15 min. After heat shock treatment, the cells were left to recover at 37°C for 10 or 60 min. Subsequently, luciferase activity in the cells was determined by One-Glo™ luciferase assay (Promega, Madison, WI) according to the manufacturer’s protocol.

Figure 2. Effect of the withanolides on the viability of MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with the indicated concentrations of various withanolide compounds for 48 h, and cell viability was determined by the MTT assay. (B) The IC_{50} values of the withanolides on the viability of MDA-MB-231 cells. Results are presented as means ± S.E.M. (n = 3).

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shRNA knockdown of Hsp70 in MDA-MB-231 cells

shRNA plasmid against Hsp70 (clone TRCN0000003945) or control plasmid (clone TRCN0000072223) were obtained from the National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taiwan. To knockdown Hsp70 expression in MDA-MB-231 cells, shRNA plasmid against Hsp70 or control plasmid was introduced into the cells using Lipofectamine 2000 (Invitrogen) according to
the manufacturer’s protocol. Forty-eight hours following transfection, the cells were treated with the withanolides.

Statistics

Data are presented as means ± S.E.M., and comparisons were made using Student’s t test or one-way ANOVA. A probability of 0.05 or less was considered statistically significant.

Results

Structure-cytotoxicity relationships of withanolides in MDA-MB-231 cells

Nine withanolides, including withaferin A (WA), tubocapsenolide A (TA), 4β-hydroxywithanolide (HW), withanolide E (WE), tubocapsanolide E (TE), anomanolide A (AA), tubocapsanolide B (TB), tubocapsanolide C (TC), and peruvianolide H (PH) (Fig. 1), were tested in the human breast cancer MDA-MB-231 cells for their cytotoxicity using the MTT assay. Fig. 2 shows that these withanolides reduced cell viability with different potency. The analysis of the structure-cytotoxicity relationships reveals that the α, β-unsaturated ketone in ring A is essential for the cytotoxicity of withanolides, since TB, TC, and PH, which lack the 2,3-unsaturated double bond, lost cytotoxic activity in MDA-MB-231 cells. Among the 2,3-unsaturated double bond-containing withanolides, WA and TA are the most potent cytotoxic compounds, and both have an additional 4β-hydroxy group and a 5β,6β-epoxide. In contrast, compounds lacking the 4β-hydroxy group (i.e., WE) or the 5β,6β-epoxide (i.e., TE) significantly reduced their cytotoxicity. These results suggest either or both of the two substitutions can enhance the cytotoxicity of withanolides. On the other hand, the addition of a hydroxyl group to C20 (i.e., HW and WE) reduces the cytotoxic effect of withanolides.

Withanolides induce apoptosis in MDA-MB-231 cells

WA, HW, AA, and PH were chosen for the further studies based on their different chemical characteristics and cytotoxicity.
In addition, the well-known Hsp90 inhibitor geldanamycin (GM) was also studied in parallel with the withanolides. We investigated if the withanolides induce apoptotic death in MDA-MB-231 cells. The activation of caspase-3, which is an important effector caspase, has been used as an indicator of apoptosis and, this process can be determined by a decrease in pro-enzyme levels using Western blot analysis. Figure 3a showed that the protein levels of procaspase-3 were decreased in MDA-MB-231 cells after treatment of WA (5 μM), HW (20 μM), AA (20 μM), or GM (10 μM) for 48 h. The activation of caspase-3 by the withanolides and GM was further confirmed by detecting the degradation of PARP, which is a DNA repair enzyme and undergo cleavage by caspase-3 during apoptosis. In contrast to other withanolides, PH induced neither caspase-3 activation nor PARP cleavage.

In order to quantify withanolide-induced apoptosis, we also performed flow cytometric analysis using annexin V-FITC/PI double staining. Annexin V has been widely used to detect the exposed phosphatidylserine on cell surface during apoptosis. When combined with PI, which stains cells that have lost membrane integrity, this approach allows a further distinction of early apoptotic (annexin V+/PI−), late apoptotic/necrotic (annexin V+/PI−) cells, and primary necrotic cells (annexin V−/PI+). Figure 3b shows that treatment of MDA-MB-231 cells with WA (5 μM), HW (20 μM), AA (20 μM), or GM (10 μM) for 48 h led to significant increases of both early and late apoptotic cells.

**Figure 5. Effect of heat shock on luciferase expressed in vivo in the presence of withanolides.** MDA-MB-231 cells expressed luciferase were pretreated DMSO or withanolides in 96-well plates for 30 min before incubation at 42°C for 15 min. After heat shock treatment, cells were left to recover at 37°C for 10 or 60 min. Luciferase activity was assayed on whole-cell lysates. Results are presented as means ± S.E.M. (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 as compared with the no heat shock group. #P < 0.05, ##P < 0.01, ###P < 0.001 as compared with the DMSO group which was recovered for 60 min after heat shock.

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Withanolides induce degradation of Hsp90 client proteins and induction of Hsp70

We next determined the effect of withanolides on protein levels of the Hsp90 client proteins Akt, Raf-1, and CDK4 in MDA-MB-231 cells. As shown in Fig. 4A and B, at the concentration of 5 μM, both WA and TA treatments markedly reduced Akt, Raf-1, and CDK4 levels. At a higher concentration (20 μM), HW, WE, TE, and AA also caused significant decreases in Raf-1 and CDK4 levels and, to a less extent, Akt levels. The effect of the withanolides on Hsp90 client proteins was accompanied by an increase in Hsp70 expression. In contrast, the non-Hsp90 client protein PI3K p85 subunit was not affected. Importantly, TB, TC, and PH, which are weakly cytotoxic withanolides, did not reduce the amounts of the Hsp90 client proteins and did not induce Hsp70 expression in cancer cells.

Hsp70 is known as an anti-apoptotic protein, and previous studies have shown that induction of Hsp70 following Hsp90 inhibitors [13,14]. In order to determine the role of Hsp70 induction on withanolide-induced apoptosis, we used shRNA to reduce Hsp70 levels in MDA-MB-231 cells. The capacity of the shRNA against Hsp70 was confirmed using Western blot (Fig. 4 C). As shown in Fig. 4D, Hsp70 knockdown enhanced the pro-apoptotic effect of WA, HW, and AA. This result suggests that like other Hsp90 inhibitors, up-regulation of Hsp70 also plays a protective mechanism against withanolide-induced apoptosis.
Withanolides inhibit the chaperone activity of Hsp90

In order to investigate if withanolides-induced degradations of Akt, Raf-1, and CDK4 were due to inhibition of the chaperone activity of Hsp90, an in vivo heat shock luciferase activity recovery assay was used. It has been reported that firefly luciferase expressed in mammalian cells denatures at high temperatures but refolds upon temperature downshift in an Hsp90-dependent manner [11]. In the present study, 293T cells transfected with firefly luciferase-expressing plasmid was heat-stressed at 42°C for 15 min. The cells were then shifted to 37°C for 10 min and 60 min to recover the luciferase activity. As shown in Fig. 5, after 10 min and 60 min recovery, the luciferase activity reaching 69% and 86% of control activity, respectively. In the presence of geldanamycin (10 μM), luciferase activity was only 49% of control levels after 60 min recovery at 37°C, indicating that Hsp90 involves in the refolding processes. Similar to geldanamycin, WA (1–5 μM), HW (5–20 μM), and AA (5–20 μM) inhibited luciferase refolding in a concentration-dependent manner. In contrast, PH did not affect the recovery of luciferase activity.

Withanolides-induced cell death and Hsp90 inhibition are prevented by N-acetylcysteine

In the previous studies, TA and WA have been reported to induce thiol oxidation and aggregation of Hsp90 by promoting the formation of intermolecular disulfide bonds [5,6]. In the present work, we found that when MDA-MB-231 cells were treated with WA, TA, HW or AA, disulfide-linked Hsp90 with higher molecular weight appeared in both the Triton-soluble fraction and the Triton-insoluble fraction, and migrated slower than the native Hsp90 in non-reducing SDS-PAGE (Fig. 6A). These results suggested that Hsp90 was oxidative-damaged and formed insoluble aggregates in cancer cells treated with the withanolides. In contrast, the less-cytotoxic withanolide PH, had no effect on the oxidation and aggregation of Hsp90.

We next used an antioxidant N-acetylcysteine (NAC), which contains a reduced sulfhydryl group, to prevent the oxidation of Hsp90. In the presence of NAC, the effects of WA, HW, and AA on Hsp90 client proteins and Hsp70 induction were abolished (Fig. 6B). Furthermore, cell death induced by these withanolides was also markedly prevented (Fig. 6C).

Effect of withanolides and geldanamycin on IKK and NF-κB

IKK kinase (IKK), a key regulator in the NF-κB pathway, has been reported to be a Hsp90 client protein [15]. In addition, the NF-κB pathway is constitutively active in estrogen receptor (ER)-negative breast cancer cells and contributes to their hormone-independent growth [16]. Therefore, we wanted to investigate the effect of withanolides on IKK and NF-κB. Fig. 7A shows that treatment of MDA-MB-231 cells with WA, HW, and AA on Hsp90 client proteins and Hsp70 induction were abolished (Fig. 6B). Furthermore, cell death induced by these withanolides was also markedly prevented (Fig. 6C).
inhibition of the NF-κB pathway by withanolides was further supported by that NF-κB-regulated gene products Bcl-2, Bcl-xL, and c-FLIP [17,18] were reduced by WA, HW, and AA. PH, which is inactive in inhibiting Hsp90, did not affect IKK levels and failed to inhibit NF-κB activation (Fig. 7B).

In addition, NAC pretreatment completely prevented the effect of withanolides on IKK and NF-κB. In contrast, NAC only partially reversed the effect of geldanamycin (Fig. 7C).

**Effect of the withanolides on ER-positive human breast cancer cells**

In order to confirm the generality of the Hsp90-inhibitory effect of the withanolides, another human breast cancer cell line MCF-7, which expresses ER, was treated with the compounds. As shown in Fig. 8A, the withanolides reduced the viability of MCF-7 cells, with potency in the order WA > HW > AA > PH. WA and HW also induced apoptotic death in MCF-7 cells as evidenced by a decrease of procaspase-7 and the cleavage of PARP (Fig. 8B). Furthermore, in correlation with their cytotoxicity, the withanolides reduced the levels of Hsp90-client proteins (Akt and Raf-1) and induced Hsp70 overexpression in MCF-7 cells (Fig. 8C). In addition, the withanolides reduced the expression of ER in MCF-7 cells (Fig. 8C), but the rank order of potency was HW ≥ WA > PH > AA.

As observed in MDA-MB-231 cells, the treatment of MCF-7 cells with the withanolides also resulted in a decrease in the levels of both IKKα and IKKβ, with the consequent decrease in the phosphorylation of IκB and NF-κB (Fig. 8D).

**Discussion**

There are several mechanisms of action proposed for withanolides, including NF-κB inhibition, PAR-4 induction, actin microfilament and/or vimentin intermediate filament aggregation, ROS formation, proteasome inhibition and Hsp90 inhibition [5,19–24]; however, it remains unclear which of them is a common property and contributes mainly to the anticancer activities of withanolides. In this work, we found that a panel of withanolides inhibited Hsp90 function in human breast cancer cells, as measured by depletion of client proteins and by chaperone activity, to different extents. Furthermore, there is a good correlation between Hsp90 inhibition and the cytotoxic effect caused by the withanolides. These results support that Hsp90 inhibition is an important mechanism of anticancer activity of withanolides.

The Hsp90-inhibitory effect of withanolides was apparent due to induction of oxidative insults of Hsp90 as shown in the previous reports [5,6], because withanolides that inhibited Hsp90 functions also induced Hsp90 oxidation and aggregation, and the thiol antioxidant NAC prevented both oxidative insults and function loss of Hsp90 caused by the withanolides. Structure-activity analysis of the withanolides in the present study indicates that the α, β-unsaturated ketone in ring A is critical for inducing Hsp90 oxidative damage. α, β-Unsaturated ketones are strong electrophilics which can react with protein cysteine residues by Michael addition that results in thiol alkylation and/or thiol oxidation [25]. Besides the α, β-unsaturated ketone, the epoxide in ring B and the conjugated ketone in the lactone ring of withanolides are also electrophilic moieties [26]. However, the electrophilicity of the conjugated ketone in the lactone ring is much lower than that of α, β-unsaturated ketone, since a methyl substitution at the β-position of the former prevents nucleophilic attack. As a result, the withanolides containing only a conjugated ketone in the lactone ring (e.g. PH) had no or little effect on Hsp90 oxidation and inhibition.

Several withanolides, including WA, have been reported to inhibit NF-κB activation in tumor necrosis factor (TNF)-stimulated cells [19,27,28]. Although the mechanisms involved in NF-κB inhibition by withanolides are not fully understood, previous studies have shown that withanolides can inhibit IkB phosphorylation and degradation with consequent reduction of NF-κB in the nucleus [27]. The IkB kinase complex contains two kinase subunits, IKKα and IKKβ, which is inactive in inhibiting Hsp90, do not affect IKK levels and failed to inhibit NF-κB activation (Fig. 7B).

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contributes to the resistance of cancer cells to chemotherapy and radiotherapy [31,32]. In human breast cancer cell lines or specimens, the NF-κB signaling is inversely related to estrogen receptor (ER) expression [16,33]. The constitutive activation of NF-κB promotes the more invasive phenotype of ER-negative cancer cells via induction of anti-apoptotic and pro-metastatic genes, such as Bcl-2 and granulocyte macrophage-colony stimulating factor (GM-CSF) [34,35]. Moreover, the inhibition of NF-κB signaling induces apoptosis in ER-negative breast cancer cells [36,37]. These findings suggest that NF-κB is a potential target for the treatment of metastatic estrogen-independent breast cancer. In the present study, we show that withanolides inhibit the constitutive activation of NF-κB in MDA-MB-231, an ER-negative human breast cancer cell line, and reduced the anti-apoptotic proteins (Bcl-2, Bcl-xL, and c-FLIP) which are regulated by NF-κB. Given the importance of NF-κB in cancer cell survival, it is suggested that the inhibition of the IKK/NF-κB pathway may contribute to the anticancer effect of withanolides.

In contrast to MDA-MB-231 cells, the survival of ER-positive breast cancer cells, such as MCF-7, are less dependent on the IKK/NF-κB pathway [37]. Therefore, the inhibition of IKK/NF-κB by withanolides may not play a major role in their cytotoxicity against MCF-7. On the other hand, the withanolides exhibit potent inhibition of ER expression, an effect potentially linked to suppression of ER-dependent proliferation of breast cancer cells [38]. The reduced ER expression by withanolides may be, at least in part, due to inhibition of Hsp90, since ER is also a Hsp90 client protein [39,40]. Of note, HW exhibits greater inhibitory effect on ER expression than that of WA suggesting that mechanisms other than Hsp90 inhibition may also contribute to its action on ER.

In conclusion, structure-activity analysis of a panel of withanolides reveals that 2,3-unsaturated double bond-containing withanolides exhibit potent inhibitory effect on Hsp90 chaperone activity. The inhibition of Hsp90 by the withanolides causes the depletion of several important signaling molecules involved in cell survival, including IKKs, and thus contributes to the anticancer effect of the withanolides.

Author Contributions

Conceived and designed the experiments: CCW HCW. Performed the experiments: YLT MHL WYC. Analyzed the data: CCW HCW. Contributed reagents/materials/analysis tools: FRC YCW. Wrote the paper: CCW HCW.
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References

1. Mishra LG, Singh BB, Dagenais S (2000) Scientific basis for the therapeutic use of Withania somnifera (ashwagandha): a review. Alerm Med Rev 5: 334–346.
2. Mirjalili MH, Moyano E, Bonfill M, Cusidó RM, Palazon J (2009) Steroidal lactones from Withania somnifera, an ancient plant for novel medicine. Molecules 14: 2573–2593.
3. Chen LX, He H, Qiu F (2011) Natural withanolides: an overview. Nat Prod Rep 28: 705–740.
4. Hsieh PW, Huang ZY, Chen JH, Chang FR, Wu CC, et al. (2007) Cytotoxic withanolides from Tubocapsicum annuum. J Nat Prod 70: 747–753.
5. Chen WY, Chang FR, Huang ZY, Chen JH, Wu YC, et al. (2008) Natural withanolides: an overview. Nat Prod Rep 25: 705–740.
6. Chen LX, He H, Qiu F, et al. (2010) Hsp90/Withanolides and inactivation of heat shock protein 90. J Biol Chem 283: 17181–17193.
7. Yu Y, Hamza A, Zhang T, Gu M, Zou P, et al. (2010) Withaferin A targets heat shock protein 90 in pancreatic cancer cells. Biochem Pharmacol 79: 542–551.
8. Mirjalili MH, Moyano E, Bonfill M, Cusidó RM, Palazoñ J (2009) Steroidal lactones from Withania somnifera. Molecules 14: 2573–2593.
9. Cheng FR, Chang FR, Huang ZY, Chen JH, Wu YC, et al. (2007) Hsp90/cdk57 chaperone/c-co-chaperone complex, a novel junction anticancer target elucidated by the mode of action of herbal drug Withaferin A. BMC Bioinformatics 12 Suppl 1: S80.
10. Yen GY, Chiu CC, Chang FR, Chen JY, Huang CC, et al. (2010) 4H-Hydroxywithanolide E from Physalis peruviana (golden berry) inhibits growth of human lung cancer cells through DNA damage, apoptosis and G2/M arrest. BMC Cancer 10: 46.
11. Cumming RC, Audon NL, Haynes PA, Park M, Fischer WH, et al. (2004) Protein disulfide bond formation in the cytoplasm during oxidative stress. J Biol Chem 279: 21547–21558.
12. Chen WY, Hsieh YA, Tsai CL, Kang YT, Chang FR, et al. (2011) Protopanaxagenine, a natural derivative of epimedium, inhibits mitogen-activated protein kinase-dependent apoptosis in human breast cancer cells associated with induction of oxidative stress and inhibition of glutathione-S-transferase π. Invest New Drugs 29: 1347–1359.
13. Schneider C, Sepp-Lorenzino L, Nimmagadda E, Ouerfelli O, Danishefsky S, et al. (1996) Pharmacologic shifting of a balance between protein refolding and degradation mediated by Hsp90. Proc Natl Acad Sci U S A 93: 14536–14541.
14. Hernández A, López-Lluch G, Bernal JA, Navas P, Pintor-Toro JA (2000) Dicumarol down-regulates human PTG1/Secerin mRNA expression through inactivation of Hsp90. Mol Cancer Ther 7: 474–482.
15. Guo F, Rocha K, Bäi P, Prampat M, Finkus W, et al. (2001) Abrogation of heat shock protein 70 induction as a strategy to increase antileukemia activity of heat shock protein 90 inhibitor 17-allylamino-demethoxy geldanamycin. Cancer Res 61: 10536–10544.
16. Powers MV, Clarke PA, Workman P (2008) Dual targeting of HSF1 and HSPl72 inhibits HSPl90 function and induces tumor-specific apoptosis. Cancer Cell 14: 250–262.
17. Brocker M, Krampmann D, Schreiter C (2004) Requirement of Hsp90 activity for IkappaB kinase (IKK) biosynthesis and for constitutive and inducible IKK and NF-kappaB activation. Oncogene 23: 5378–5386.
18. Nakahari H, Hatt N, Nakahari P, Martin DA, Goulet RJ Jr., Slodce GW Jr. (1997) Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. Mol Cell Biol 17: 3629–3639.
19. Kurea S, Siegmund M, Scherlich P, Wajant H (2001) NF-kB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. Mol Cell Biol 21: 3964–3973.
20. Bharti AC, Aggarwal BB (2002) Nuclear factor-kappa B and cancer: its role in prevention and therapy. Biochem Pharmacol 64: 759–866.
21. Kailath M, Vanden Berghe W, Heyerick A, Horion J, Pette J, et al. (2007) Withaferin A strongly elicits I kappaB kinase (IKK) biosynthesis and for constitutive and inducible NF-kappaB activity in human breast cancer specimens and its role in cell proliferation and apoptosis. Proc Natl Acad Sci U S A 101: 10137–10142.
22. Van Waes C (2007) Nuclear factor-kappaB in development, prevention, and therapy of cancer. Clin Cancer Res 13: 1076–1082.
23. Prasad S, Ravindran J, Aggarwal BB (2010) NF-kB and cancer: how intimate is this relationship. Mol Cell Biochem 336: 25–37.
24. Biswas DK, Shi Q, Baily S, Strickland I, Ghosh S, et al. (2004) NF-kB activation in human breast cancer specimens and its role in cell proliferation and apoptosis. Mol Cancer Res 6: 894–902.
25. Biswas DK, Cruz AP, Gansberger E, Pardee AB (2000) Epidermal growth factor-induced nuclear factor-kappa B activation: A major pathway of cellcycle progression in estrogen-receptor negative breast cancer cells. Proc Natl Acad Sci U S A 97: 8542–8547.
26. Castoria G, Micali A, Giovannielli P, Antonucci F (2010) Cell proliferation regulated by estradiol receptor: Therapeutic implications. Steroids 75: 524–527.
27. Sengupta B, Gehring U (1997) The function of steroid hormone receptors is destabilized by heat shock protein 90-binding drugs. J Biol Chem 272: 18755–18761.
28. Bagatell R, Khan O, Paine-Murrieta G, Taylor CW, Akinaga S, et al. (2001) Destabilization of steroid receptors by heat shock protein 90-binding drugs: a ligand-independent approach to hormonal therapy of breast cancer. Clin Cancer Res 7: 2076–2084.