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### Permalink

<https://escholarship.org/uc/item/4qd7d2mq>

### Journal

Molecular Carcinogenesis, 59(2)

### ISSN

0899-1987

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### Publication Date

2020-02-01

### DOI

10.1002/mc.23140

Peer reviewed



Published in final edited form as:

*Mol Carcinog.* 2020 February ; 59(2): 193–201. doi:10.1002/mc.23140.

## Gartanin is a novel NEDDylation inhibitor for induction of Skp2 degradation, FBXW2 expression and autophagy

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### Abstract

Gartanin, a 4-prenylated xanthone, has been identified from the purple mangosteen fruit as a potent growth inhibitor of various cancer cell lines, including prostate cancer. However, much of Gartanin's anti-cancer mechanism remains unknown. We have discovered that Gartanin docked onto the regulatory subunit of the precursor cell-expressed developmentally down-regulated 8 (NEDD8)-activating enzyme (NAE) complex and next to the NEDD8 binding complex, which leads to inhibit NEDD8 conjugations to both Cullin1 and Ubc12 in prostate cancer cell lines and Ubc12 NEDDylation in an *in vitro* assay. S phase kinase-associated protein (Skp2) and F-box and WD-repeat domain-containing 2 (FBXW2), the NEDD8 family members of E3 ubiquitin ligases, were also down-regulated and up-regulated by Gartanin, respectively. Knock-down of NEDD8 expression by short harpin (Sh) RNAs blocked or attenuated these effects of Gartanin. Finally, Gartanin demonstrated its ability to inhibit growth of prostate cancer lines *via* autophagy initiation. Our data support that Gartanin is a naturally occurring NEDDylation inhibitor and deserves further investigation for prostate cancer prevention and treatment.

### Keywords

Gartanin; Skp2; neddylation; chemoprevention; prostate cancer

## INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer in men. There will be an estimated 31,620 deaths from prostate cancer in the United States in 2019, a 7% increase from 2018 [1]. Prostate cancer is a highly heterogeneous disease. The course of prostate cancer is difficult to predict. Prostate cancer slowly progresses over several decades and in a significant proportion of elderly men with prostate cancer, the disease never becomes symptomatic [2]. This group of patients is easily over-treated and suffered from unnecessary

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of Interest Disclosures: The authors declare no potential conflicts of interest.

adverse effects of treatments and loss of quality of life. In addition, between 15% and 50% of men who are thought to have clinically localized disease at diagnosis have failure in conventional therapy leading to a lethal disease. Based on these characteristics, prostate cancer has been considered to an ideal target for prevention by chemo/dietary approaches.

Fruits and vegetables contain a wide variety of new anti-cancer agents. *Garcinia mangostana L(mangosteen)*, also called “The Queen of Fruits”, is one of the most popular tropical fruits widespread in Southeast Asian countries [3]. Mangosteen has been used as food and folk medicine by Southeast Asians for centuries [3] and sold as a health drink or dietary supplements in the United States. Mangosteen is rich in oxygenated and prenylated xanthenes. Studies from cell cultures and animals have demonstrated that these xanthenes have antioxidant, antitumor, antiallergic and anti-microbial activities [3–7]. Among these mangosteen xanthenes, Gartanin has been identified to the most potent agent against the growth of bladder, breast, prostate and colon cancer, sarcoma, glioma, melanoma and leukemia cell lines *via* cell cycle arrest and apoptosis induction [8–15]. Molecular mechanisms of Gartanin’s anti-cancer effects were involved in inhibition of JNK, AKT, MAPK, and NF- $\kappa$ B pathways and CDK4 [9, 12, 14, 15]. In addition, our previous study has demonstrated that Gartanin induces autophagy through the inhibition of the mTOR pathway [10]. In prostate cancer, Gartanin has been shown to interact with androgen receptor and induce its degradation through the unfolded protein response and endoplasmic reticulum stress pathway [13]. However, the mechanism of Gartanin-induced protein degradation is still largely unclear.

NEDD8, an ubiquitin-like protein, plays an important role in the modification of Cullin-1 to turn on the Skp1-Cullin-F box protein (SCF) complex for regulation of the stability of its target proteins. The NEDDylation of Cullin1 occurs via a conjugation cascade-the NEDDylation pathway, which is initiated by an E1 (*i.e.* NAE) enzyme. Activated E1 then transfers NEDD8 to its E2 enzyme NEDD8-conjugating enzyme 2M (UBE2M), also called Ubc12, which causes covalent modulation of Cullin proteins with NEDD8 for activation of Cullin-RING ubiquitin ligases. Many components of the NEDDylation pathway, such as NEDD8 and NAE1, have been reported to be over-expressed in several cancers [16–18]. Currently, the knowledge about a role of NEDDylation in prostate cancer remains largely unclear. Limited studies have shown that the expression of the CUL1-interacting protein cullin-associated and neddylation-dissociated 1 (CAND1) and defective in cullin neddylation 1 (DCN1) that regulate the conjugation of NEDD8 to Cullins were aberrantly altered in prostate cancer [19, 20]. In addition, it has been thought that NEDDylation play an important role in prostate cancer by regulation of the equilibrium of cellular E3 ubiquitin ligase repertoire, such as Skp2, SPOP, etc., leading to enhanced survival of cancer cells and tumorigenesis [21, 22]. Therefore, the NEDDylation pathway could be targeted for development of novel cancer therapies. Indeed, a small molecule inhibitor of NAE, MLN4924 (a first-in-class inhibitor of NAE also named as pevonedistat), has been developed and currently in multiple phase I/II clinical trials for patients with advanced solid tumors or hematological tumors [23–27].

In this study, we discovered that Gartanin inhibits protein NEDDylation both *in vitro* and *in vivo* in prostate cancer cell lines (*i.e.* 22Rv1 and PC3) by interacting with the regulatory

subunit of NAE1 and NEDD8, which results in down-regulation of Skp2 and Cdh1 and up-regulation of FBXW2, as well as induction of autophagy and growth inhibition.

## MATERIALS AND METHODS

### Cell lines and reagents

PC3 and 22Rv1 were obtained from American Type Culture Collection (ATCC, Manassas, VA). These cells were characterized and authenticated by ATCC. In addition, all cell lines were tested for known species of mycoplasma contamination using a kit from LONZA Inc. (Walkersville, MD). These prostate cancer cells were cultured in RPMI-1640 media (Fisher Scientific) supplemented with 10% fetal bovine serum (FBS), 1% L-Glutamine, and 1% penicillin-streptomycin as described previously in our publication. The NEDD8 knockdown and Skp2 transfected cell lines were obtained according to our reported methods [28, 29].

### Compounds, antibodies and reagents

Gartanin was obtained from Chromadex with 99% purity (Irvine, CA). MG-132 and MLN4924 were obtained from Cayman Chemical Inc. (Ann Arbor, MI). Antibodies against Ubc12, ubiquitin, and  $\beta$ -tubulin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Skp2 antibodies were purchased from Invitrogen (Grand Island, NY). Anti-Myc-tag, anti-LC3B-II, anti-FBXW2, anti-Cdh1, and anti- $\beta$ -TrPC antibodies were from Cell Signaling Inc. (Boston, MA). Anti-Cullin-1, anti-FBW7 and anti-NEDD8 antibodies were from Abcam (Cambridge, MA). Anti-p27/Kip1 antibody was from BD Biosciences (Billerica, MA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma. Reverse Transcription System kit and was from Promega (Mandison, WI). A quantitative reverse transcription polymerase chain reaction (RT-PCR) kit was from Bio-Rad (Hercules, CA).

### MTT assay

Cells ( $2 \times 10^4$  cells/well) were grown in 24-well culture dishes for 24 hours, and then treated as indicated in the figures. Cells were then incubated for another 72 hours before adding 1 mg/mL MTT in 20% PBS and 80% culture medium (v/v) for 2 hours. The absorbance was read at 570 nm, and the dose-response curves for reduction of cell viability were generated as percentage ratios of vehicle-treated controls.

### Western blotting analysis

The treated cells were collected and lysed in RIPA buffer contained a protease inhibitor cocktail for 30 minutes followed by centrifugation at 15000 rpm for 30 minutes at 4 °C. After the collection of supernatants, the protein concentrations were detected. Clarified protein lysates (60–100  $\mu$ g) were denatured in 2X loading buffer at 100 °C prior to adding into 8–16% SDS-PAGE. For non-denatured Western blotting analysis, non-reducing loading buffer were mixed with protein lysates prior to resolving it in non-denaturing gel (Biorad, CA). Proteins were transferred to nitrocellulose membranes, probed with indicated antibodies, and visualized by an enhanced chemiluminescence detection system. The western blotting bands were semi-quantified using Image J and adjusted for loading controls,  $\beta$ -actin or tubulin.

### ***In vitro* NEDD8 initiation conjugation assay**

The NEDD8 initiation conjugation assay kit was purchased from Boston Biochem Inc. (Cambridge, MA). A master mix of 0.4  $\mu\text{M}$  APP-BP1/UBA3 (NEDD8 ligase E1), 12.5  $\mu\text{M}$  UbcH12 (NEDD8 ligase E2) and 62.5  $\mu\text{M}$  NEDD8 were prepared in the reaction buffer (pH 8.0, 50 mM HEPES and 50 mM NaCl in final reaction) and distributed to individual tubes with a volume of 15  $\mu\text{l}$ . A series of dilutions of Gartanin were made in DMSO. One microliter of Gartanin or DMSO was added to the indicated tubes and mixed well. The reactions were started by adding 2.5 mM  $\text{Mg}^{2+}$  and 1 mM ATP (4  $\mu\text{l}$  in mixture), except the negative control tube was added by equal volume of ddH<sub>2</sub>O. The reaction tubes were incubated in 37 °C for 30 minutes and stopped by adding 5  $\mu\text{l}$  25 mM EDTA. Non-reducing western blot was performed with anti-Ubc12 antibody to detect both Ubc12 bands and NEDD8 conjugated Ubc12 bands.

### **Cell autophagy imaging**

PC3 cell lines were stably transfected with pcDNA3-LC3-eGFP tagged vector. They were then treated for 24 hours with indicated concentration of Gartanin in a 24-well plate before imaging under a fluorescence microscope. Image J cell counter program was used to determine the number of fluorescence cells.

### **Molecular modeling studies**

Molecular modeling was performed using the autodock 4 software from the Scripps Research Institute. Gartanin as first generated using Pymol software. Following generation, the files were converted to the. pdbqt format using Open Babel. Gartanin were then docked using AutoDockTools with NAE1 protein (PDB code: 3dbl) from MGLTools their resulting conformations were visualized using Pymol.

### **Statistical evaluation**

Data were presented as means  $\pm$  SD. Statistical analyses were performed by the analysis of variance (ANOVA). All statistical analyses were performed by SPSS 17.0 and Excel.

## **RESULTS**

### **Gartanin inhibits *in vitro* neddylation.**

We have screened natural compounds for their inhibitory activity against NEDDylation and Gartanin was identified as a potent NEDDylation inhibitor (Data not shown). Autodock program was used to dock Gartanin with NAE1 protein (PDB code: 3dbl), which has a predicted IC<sub>50</sub> value of 735 nM, and Pymol program was used to observe the superimposed binding of Gartanin to the regulatory subunit of NAE1 and next to NEDD8 (Fig. 1A). Gartanin was predicted to form multiple hydrogen bonds with the NAE1 regulatory subunit and with NEDD8 within the docking site (Fig. 1B).

Furthermore, *in vitro* Ubc12 NEDDylation initiation assay was performed by using Ubc12 antibody to detect the changes of NEDD8-conjugated Ubc12 bands by the indicated concentrations of Gartanin and negative control (with no addition of ATP) as shown in the Fig. 1.C. Densitometry analysis of the Western blotting results revealed a dose dependent

decrease of Ubc12 NEDDylation by Gartanin treatment with an estimated  $IC_{50}$  value of  $\sim 10.33\mu\text{M}$  (Fig. 1D).

### **Gartanin inhibits *in vivo* NEDDylation in prostate cancer PC3 and 22Rv1 cells.**

Western blotting analysis by anti-NEDD8 antibody shows that Gartanin inhibited the NEDDylation pathway by reducing the expression levels of NEDDylated Cullin1, NAE1, UBA3, and UBE2M, as well as protein levels of NEDD8 in a concentration-dependent manner in both 22Rv1 cells and PC3 cells (Fig. 2A). Using anti-Cullin1 and Ubc12 antibodies, we further confirmed that the NEDDylation of Cullin1 and Ubc12 was decreased by Gartanin treatment in a dose and time dependent manner (Fig. 2B and C), respectively. These results support that Gartanin is a cell-active NEDDylation inhibitor.

### **Gartanin induces proteasome dependent Skp2 degradation**

Skp2 is a critical oncogene and overexpressed in human prostate cancer [30]. Skp2 acts as a down-stream E3 ligase of the NEDDylation pathway for ubiquitination of tumor suppressors, such as CDK inhibitor p27/Kip1, for targeted degradation by proteasome [31]. We found that Gartanin treatment of 22Rv1 and PC3 cells for 24 hours dose-dependently down-regulated the protein expression of Skp2 and up-regulated the expression of p27/Kip1 in both 22Rv1 and PC3 cells (Fig. 3A).

When PC3 cells were treated with  $10\mu\text{g/mL}$  cyclohexamine to inhibit *de novo* protein synthesis, Gartanin treatment at a concentration of  $12\mu\text{M}$  accelerated the decrease in protein levels of Skp2 over time compared to control treatment, suggesting that Gartanin reduces the protein stability of Skp2 (Fig. 3B&C). MG132, which acts as a blocker in ubiquitin-proteasome pathway that is involved in  $>80\%$  of intracellular protein degradation [32]. As shown in the Fig. 3D,  $12\mu\text{M}$  Gartanin alone obviously downregulated the protein expression of Skp2 in PC3/Skp2 cells. However, the combination of  $3\mu\text{M}$  MG132 and  $12\mu\text{M}$  Gartanin reversed the effect of Gartanin on Skp2 down-regulation. This result suggests that Skp2 degradation by Gartanin was due to a proteasome-dependent manner.

### **The inhibitory effects of Gartanin on Skp2 protein expression at least in part require the expression of NEDD8 but not FBXW2, Cdh1 and $\beta$ -TrCP1**

Several E3 ligases, such as FBXW2 and Cdh1, were reported to recognize Skp2 and cause Skp2 ubiquitination for targeted degradation by proteasome [33, 34]. Fig.4A shows that Gartanin dose-dependently increased the protein expression of FBXW2 and decreased the expression of Cdh1 in both 22Rv1 and PC3 cells. However, siRNA knock-down of FBXW2 did not result in an increase in the expression Skp2 under Gartanin treatment (Fig.4B). These results suggest that Cdh1 and FBXW2 expression is not required for Gartanin induced Skp2 degradation.

However, knocking-down the expression of NEDD8 by shRNAs decreased protein NEDDylation levels as detected by Western blotting analysis using anti-NEDD8 antibody (Fig. 4C), which completely blocked the Gartanin induced Skp2 protein degradation at concentrations of up to  $12\mu\text{M}$  (Fig. 4D). Knock-down of NEDD8 also blocked the effect of

Gartanin on induction of FBXW2 expression, whereas other E3 ubiquitin ligases, Cdh1, FBW7 and  $\beta$ -TrCP were less or not affected by NEDD8 knock-down.

Taken together, our results indicate that Skp2 protein down-regulation is dependent on NEDD8 expression and that inhibition of neddylation by NEDD8 knock-down may regulate the protein expression or stability of specific groups of E3 ubiquitin ligases.

### **Growth inhibitory effects of Gartanin are associated with expression of NEDD8 and Skp2 expression but not with expression of FBXW2.**

Fig. 5A demonstrates that NEDD8 knock-down also attenuated the growth inhibitory effect of Gartanin in PC3 cells. The  $IC_{50}$  values of Gartanin on the cell growth of PC3/vector control (ShLuc) and PC3/shNEDD8 cells were estimated to  $4.91 \pm 0.13 \mu\text{M}$  and  $14.11 \pm 0.16 \mu\text{M}$  ( $P < 0.05$ ), respectively. In addition, PC3 cells with stable overexpression of Skp2 are more sensitive to Gartanin treatment than those PC3 cells transfected with vector (PcDNA3) control with estimated  $IC_{50}$ s of  $3.55 \pm 0.31 \mu\text{M}$  and  $9.22 \pm 0.10 \mu\text{M}$  ( $P < 0.05$ ), respectively (Fig. 5B).

However, siRNA knock down of FBXW2 expression did not significantly change the growth inhibitory effects of Gartanin on PC3 cells ( $P > 0.05$ ) (Fig. 5C). The  $IC_{50}$  values of Gartanin against the growth of parental 22Rv1 and PC3 cells were estimated to be about  $8.32 \pm 0.18 \mu\text{M}$  and  $13.56 \pm 0.20 \mu\text{M}$ , respectively (Fig. 5D).

These results suggest that Gartanin target NEDDylation and Skp2 for its growth inhibitory effects on prostate cancer cell lines.

### **Gartanin induces autophagy in prostate cancer cells.**

The autophagic effect of Gartanin on prostate cancer cells have not been reported yet. To determine whether Gartanin induces autophagy in prostate cancer cells, we established PC3 cell line with stable expression of pEGFP-LC3 to observe fluorescent LC3B protein. As shown in the Figs. 6A and B, the punctate staining of LC3B significantly increased after 24 hours of treatment with Gartanin (6 $\mu\text{M}$ , 12 $\mu\text{M}$  and 24 $\mu\text{M}$ ) compared with vehicle control (0.1% DMSO) treatment. In addition, we observed accumulation of cleaved LC3 induced by Gartanin using Western Blotting analysis in 22Rv1 and PC3 cells (Fig. 6C), further confirming the initiation of autophagy by Gartanin.

In summary, the mechanism of Gartanin's action can be simply depicted as in Fig. 6D. Gartanin act as a novel neddylation inhibitor for removing NEDD8 from Ubc12 and Cullin-1, which changes the cellular repertoire of specific SCF ubiquitin ligases, including Skp2, FBXW2, and others and eventually leads to cell growth inhibition and induction of autophagy.

## **DISCUSSION**

Defects in the NEDD8 cycle leading to increased protein NEDDylation have been reported in several cancers, including lung adenocarcinomas, squamous-cell carcinoma, and hepatocellularcarcinoma, and thus the NEDDylation pathway has been considered as a

major target for cancer intervention [19]. For the first time, we have provided evidence that Gartanin is a cell active NEDDylation inhibitor by inhibiting Ubc 12 and Cullin1 NEDDylation *in vivo* in prostate cancer cell lines and Ubc12 NEDDylation *in vitro*. Our results suggest that Gartanin is distinct from MLN-4294, a known NEDDylation inhibitor currently in clinical trials, which has been shown to increase the protein expression of the NEDD8 family of E3 ubiquitin ligases, such as Skp2 and FBXW2 [33]. In addition, MLN-4294 forms NEDD8-MLN-4294 adduct for inhibition of the NAE enzyme activity, whereas Gartanin is predicted to bind to the E1 regulatory subunit of the NAE.

The NEDD8 family of E3 ubiquitin ligases, such as Skp2, FBXW2, Cdh1, FBW7, and  $\beta$ -TrCP1, that act as down-stream events of the NEDDylation pathway, play a critical role in cell cycle regulation, cellular senescence, autophagy, apoptosis, metabolism, metastasis, and cancer stem cells by ubiquitinating a wide range of substrates for targeted degradation by the proteasome [33–37]. The gain or loss of function of these E3 ubiquitin ligases has been shown to contribute to tumorigenesis [33–37]. Therefore, these E3 ligases also represent promising targets for therapeutic manipulation [38]. The results from our experiments support the hypothesis that Gartanin is a novel NEDDylation inhibitor that can affect the equilibrium of cellular E3 ubiquitin ligase repertoire leading to its anti-proliferative effects. In particular, we have shown that both down-regulation of Skp2 expression and up-regulation of FBXW2 by Gartanin requires the expression of NEDD8 in prostate cancer cells (Fig. 4D).

Skp2 is a critical oncogene in human prostate cancer [39]. Skp2 was found to be overexpressed in high-grade prostatic intra-epithelial neoplasia lesions, primary, and metastatic prostate cancer [39]. In addition, Skp2 expression has been shown to be required for spontaneous tumor development that occurs in the pRb, the p19Arf and Pten deficient mouse models [40, 41]. More recently, multiple components of the SCF<sup>SKP</sup> Cullin F box containing complex including Skp2 has been identified to be candidates of highly penetrant, synthetic lethal interactions in RB defective triple negative breast cancer [42]. It is thus of import that new agents that attenuate Skp2 expression are discovered. We have demonstrated Gartanin, while acting primarily as a NEDDylation inhibitor, can selectively degrade Skp2 in prostate cancer cells.

FBXW2, a newly reported tumor suppressor, inhibits the growth, migration and invasion of lung cancer cell lines [33, 37]. FBXW2 functions as an E3 ligase to recognize Skp2 and  $\beta$ -catenin for targeted degradation [33, 37]. However, the function role and expression of FBXW2 in prostate cancer remains unknown and no reports discovered that natural products could affect FBXW2 expression in prostate cancer cells. In this work, Gartanin as a dietary xanthone from *Garcinia mangostana L.* increases the protein levels of FBXW2 in a concentration dependent manner against 22Rv1 and PC3 cells. However, the knock-down of FBXW2 did not attenuate the inhibitory effects of Gartanin on Skp2 expression and cell growth (Fig. 4B and Fig. 5C). Therefore, the contribution of FBXW2 expression in the anti-prostate cancer effects of Gartanin remains unclear and requires further investigation, which is beyond the scope of this manuscript.



In conclusion, many patients with prostate cancer are currently being over-treated and suffered from unnecessary adverse effects of treatments and loss of quality of life [2]. Dietary Gartanin, a polyphenolic xanthone that was isolated from the pericarp and whole fruit of mangosteen, has the potential of low toxicity. Dietary Gartanin may have a novel use as a NEDDylation inhibitor for selective degradation of oncogene Skp2 and up-regulation of tumor suppressor FBXW2 leading to induction of autophagy and growth inhibition of prostate cancer cells. Future studies should attempt to determine the *in vivo* efficacy and pharmacokinetics characteristics of dietary Gartanin for preventive intervention of prostate carcinogenesis in animal models.

### Acknowledgement:

This work was supported in part by NIH award 1R01CA193967-01A1 and 1R21CA152804-01A1 (to X. Zi.). Victor Pham is currently supported by NSF graduate research fellowship program DGE-1321846.

### Abbreviations:

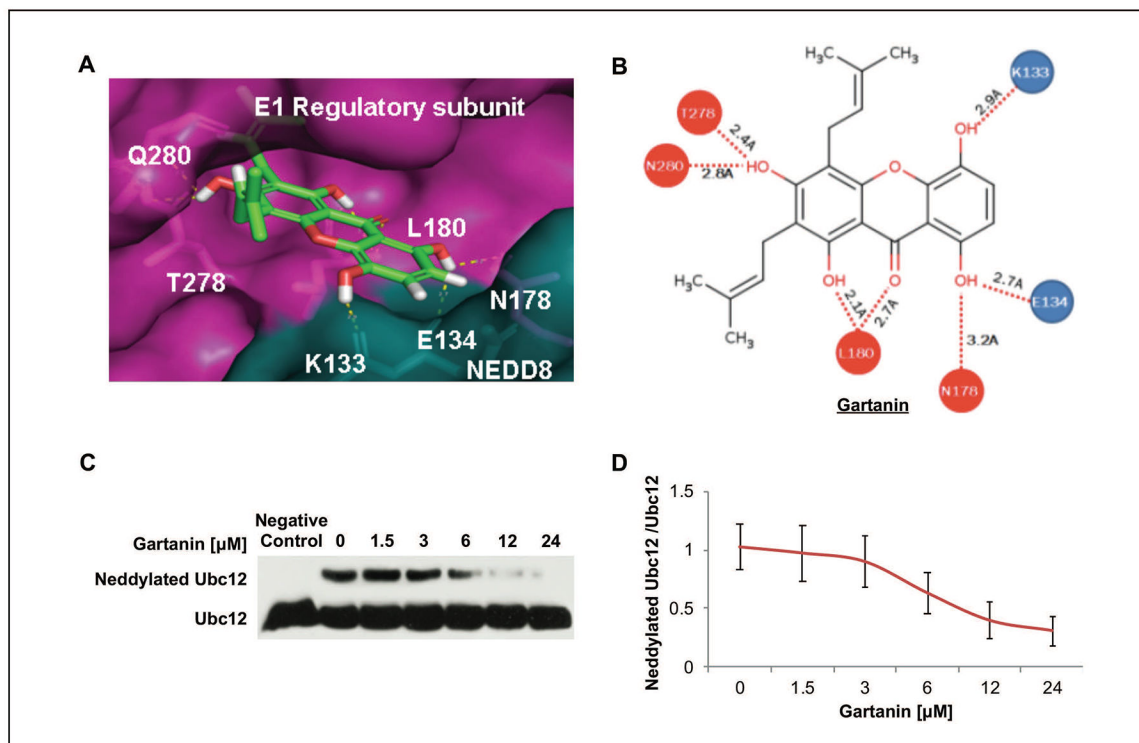
<b>NEDD8</b>	the precursor cell-expressed developmentally down-regulated 8
<b>NAE</b>	NEDD8-activating enzyme
<b>Skp2</b>	S phase kinase-associated protein
<b>ShRNAs</b>	short harpin RNAs
<b>UBE2M or Ubc12</b>	NEDD8-conjugating enzyme 2M
<b>FBXW2</b>	F-box and WD-repeat domain-containing 2

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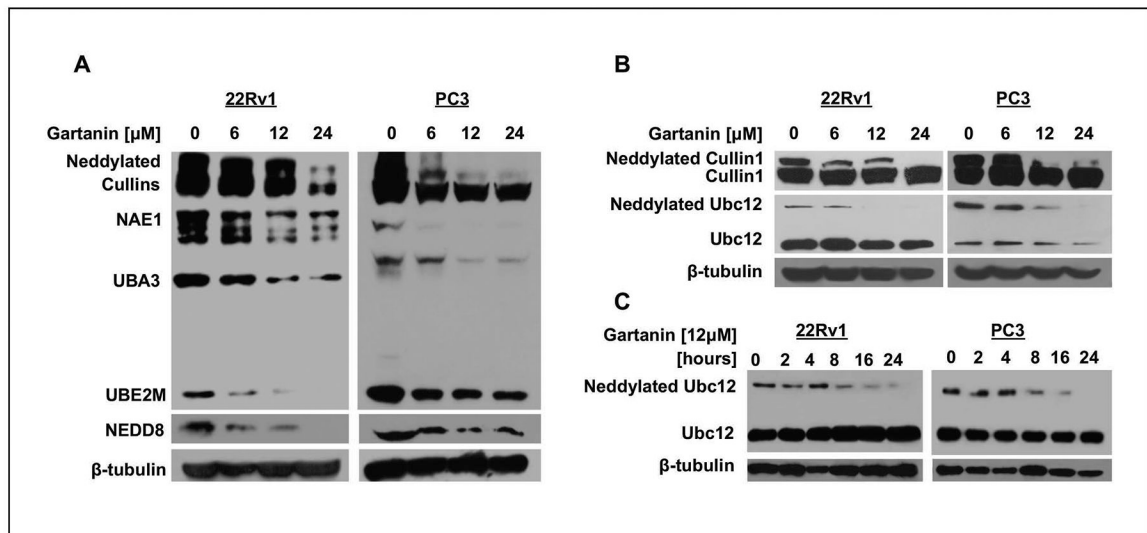
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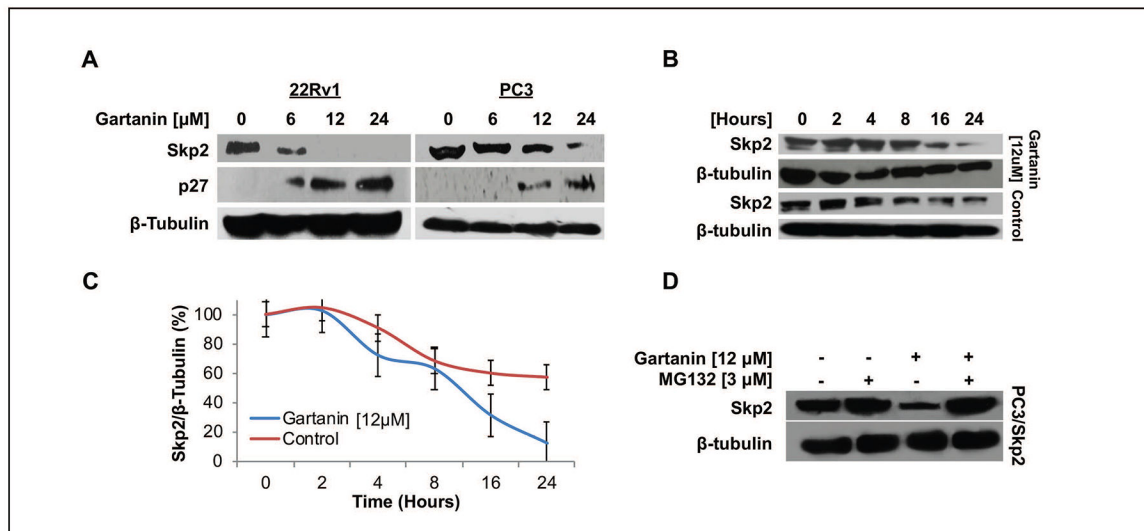
**Fig. 1. Gartanin inhibits NEDDylation *in vitro*.**

**A.** Autodocktools program was used to dock Gartanin with NAE1 protein, which has a predicted  $IC_{50}$  of 735 nM, and Pymol program was used to observe the superimposed binding of Gartanin to NAE1. Gartanin was predicted to bind within the E1 regulatory subunit (Pink) of NAE1 and interacts with NEDD8 (Blue). **B.** 2D molecular structure of Gartanin is shown to form hydrogen bonds with the NAE1 regulatory subunit (Red) and NEDD8 (Blue) within the predicted binding site. **C.** *In vitro* Ubc12 NEDDylation initiation assay was performed. Negative control contains no addition of ATP. **D.** The ratio of neddylated Ubc12 compared with Ubc12 from above Western blotting analysis results.



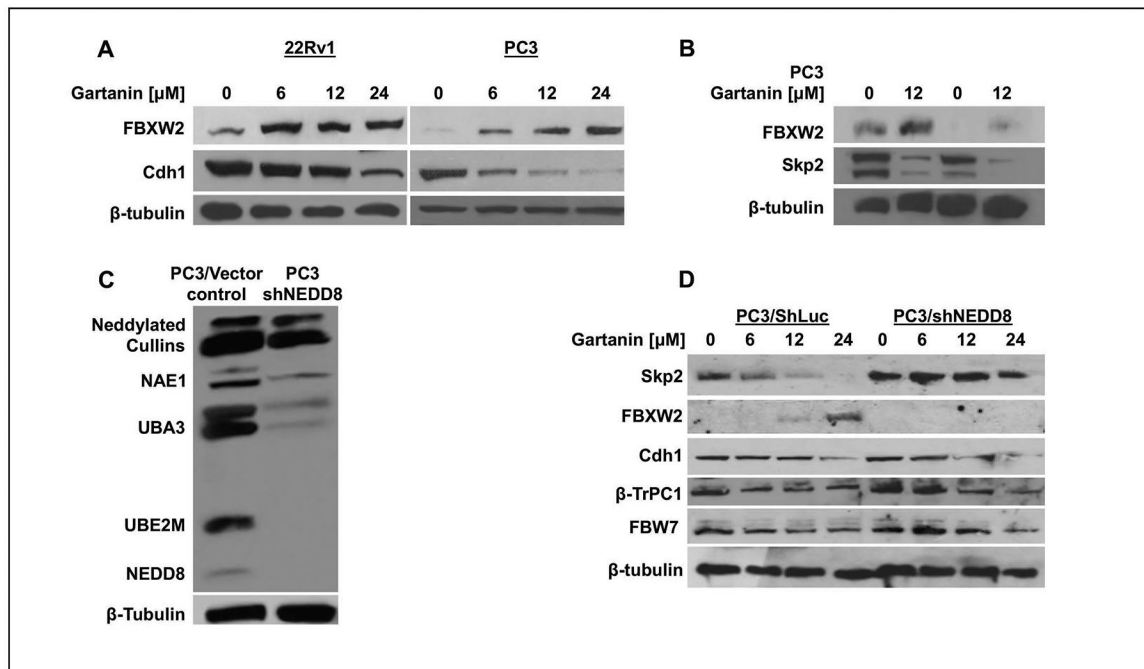
**Fig. 2. Gartanin inhibits NEDDylation of Cullin1 and Ubc12 in prostate cancer PC3 and 22Rv1 cells.**

**A.** The expression of neddylated proteins (Cullin1, Ubc12, NAE1, UBA3, UBE2M and NEDD8) in PC3 and 22Rv1 cells treated with vehicle control (0.1% DMSO) or indicated concentrations of Gartanin for 24 hours. **B.** Western blotting analysis of neddylated Cullin1 and Ubc12 protein levels in PC3 and 22 Rv1 cells treated with indicated concentrations of Gartanin for 24 hours. **C.** Western blotting analysis of neddylated Ubc12 protein levels after 22Rv1 cells were treated with 12  $\mu$ M Gartanin for different times.



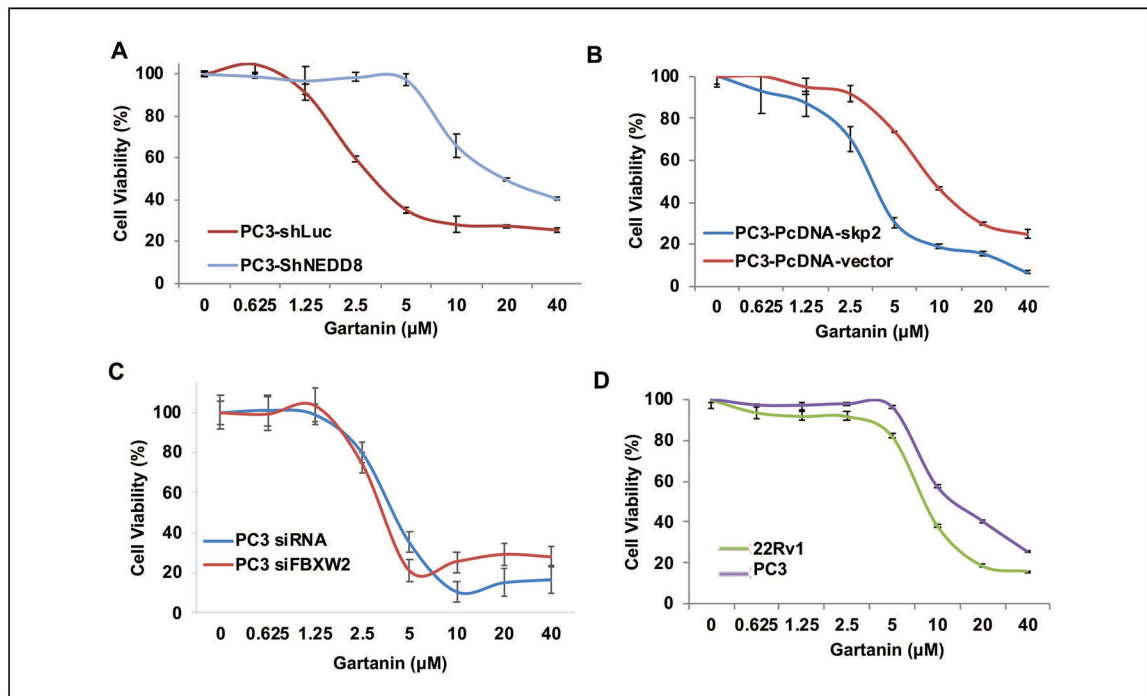
**Fig. 3. Gartanin induces Skp2 proteasome-dependent degradation.**

**A.** The expression of Skp2 and p27 in PC3 and 22Rv1 cells after treatment with vehicle control (0.1% DMSO) or indicated concentrations of Gartanin for 24 hours. **B.** PC3 cells were treated with 10  $\mu$ g/mL cyclohexamine for 8 hours to inhibit *de novo* protein synthesis, followed by 0.1% DMSO or 12  $\mu$ M Gartanin treatment for 0, 2, 4, 8, 16, and 24 hours in order to follow up changes of Skp2 protein stability. **C.** Skp2 protein levels over time were semi-quantified by densitometry measurement of band densities using image J software. **D.** PC3/Skp2 cells were treated with 3  $\mu$ M MG132 (proteasome inhibitor), 12  $\mu$ M Gartanin, or a combination treatment of MG132 and Gartanin for 24 hours. Myc-Skp2 protein expression was observed under Western blotting analysis with anti Myc-tag antibody.



**Fig. 4. Gartanin induced Skp2 degradation at least in part requires the expression of NEDD8 protein.**

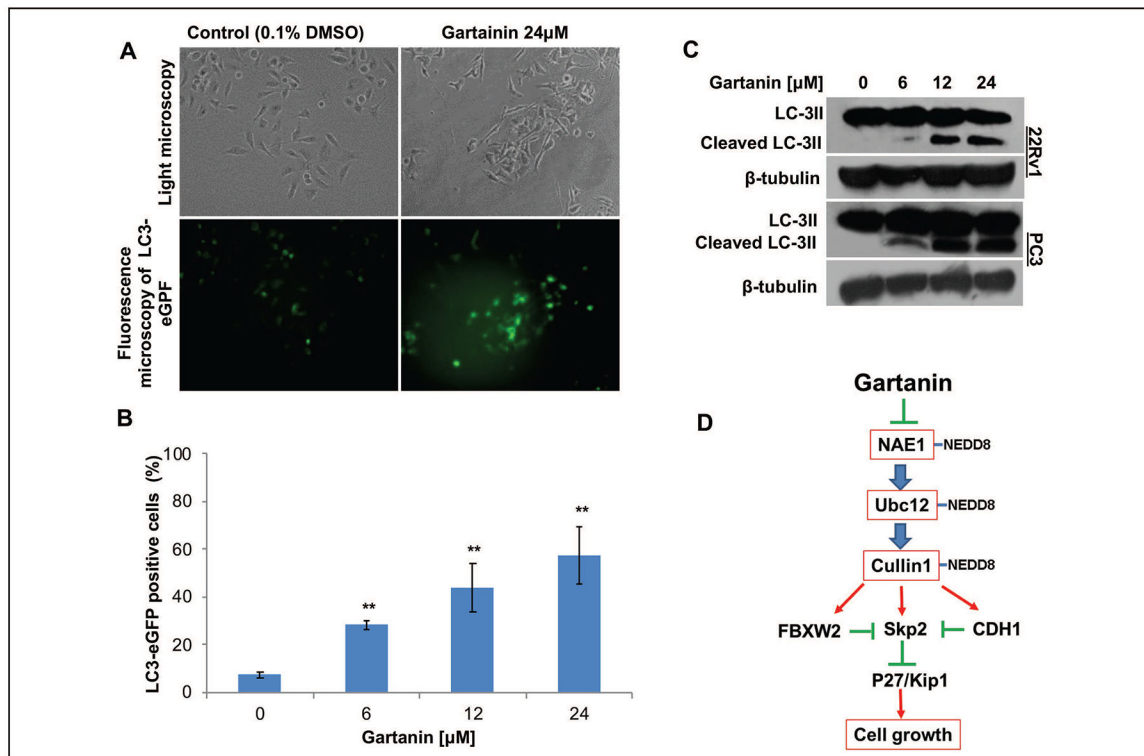
**A.** The expression of E3 ligases FBXW2 and Cdh1 were analyzed by Western blotting after treatments with 0.1% DMSO or indicated concentrations of Gartanin for 24 hours in 22Rv1 and PC3 cells. **B.** PC3 cells were transfected with siRNA controls or FBXW2 siRNAs for 24 hours and then treated with 0.1% DMSO or 12  $\mu$ M gartanin for additional 24 hours. Western blotting analysis of protein levels of FBXW2 and Skp2 is shown. **C.** Expression of NEDD8 and neddylated proteins was analyzed in PC3/ShLuc and PC3/shNEDD8 knockdown cells. **D.** The expression of E3 ligases, Skp2, FBXW2, Cdh1, FBW7, and  $\beta$ -TRCP, in PC3/ShLuc and PC3/shNEDD8 knockdown cells treated with 0.1% DMSO or indicated concentrations of Gartanin.



**Fig. 5. The growth inhibitory effects of Gartanin is, at least in part dependent, on the expression of Skp2 and NEDD8.**

Growth inhibitory effects of Gartanin in different cell lines as described below were examined by MTT assay after treatments with 0.1% DMSO or indicated concentrations of Gartanin for 72 hours. **A.** PC3/ShLuc and PC3/shNEDD8 knockdown cell lines, **B.** PC3/PcDNA3 vector control and PC3/Skp2 cell lines. **C.** PC3 cells after transfected with siRNA control and FBXW2 siRNAs for 24 hours. **D.** PC3 and 22Rv1 cell lines.





**Fig. 6. Gartanin induces autophagy.**

**A.** PC3-LC3-eGFP cells were treated with 0.1% DMSO or indicated concentrations of Gartanin for 24 hours, and then observed under a fluorescence microscope. **B.** Quantitative analysis of cells with the punctate staining of LC3B after 24 hours of treatment with indicated concentrations of Gartanin compared with 0.1% DMSO treatment. “\*\*” represents  $P < 0.01$ . **C.** 22Rv1 cells and PC3 cells were treated with indicated concentrations of Gartanin for 24 hours and LC3B antibody were used to detect LC3 cleavage. **D.** Proposed mechanisms of Gartanin’s action as a novel NEDDylation inhibitor.