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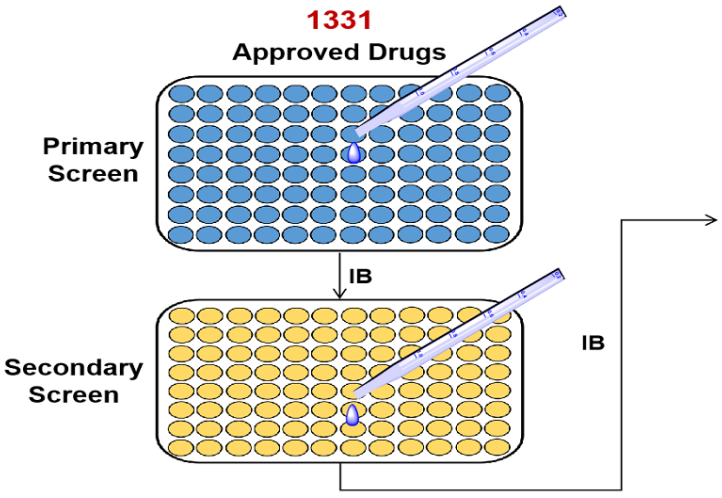
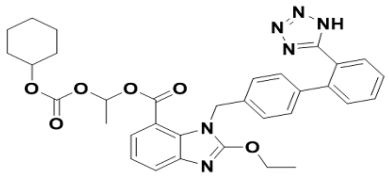


Lead Compound



candesartan cilexetic (CDC, 1)

- Inhibiting the cullin1-Nedd8 at the rang of 12.5~25 μM in enzyme-based assay;
- Inhibiting the cullins-Nedd8 at the rang of 25~50 μM in cell-based assay;
- Inhibiting various types of cacer cells and inducing the apoptosis;
- suppressing the tumor growth comparable to MLN4924 in vivo.

Discovery of candesartan cilexetic as a novel neddylation inhibitor for suppressing tumor growth

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Abstract

Protein neddylation is a posttranslational modification of conjugating the neuronal precursor cell-expressed developmentally down-regulated protein 8 (Nedd8) to substrates. Our previous work revealed that neddylation pathway is overactivated in various human lung cancers and correlates with the disease progression, whereas pharmacologically targeting this pathway has emerged as an attractive therapeutic strategy. As a follow-up research, 1331 approved drugs were investigated the inhibitory activities of cullin1 neddylation for screening the hit compounds via an improved enzyme-based assay. An antihypertensive agent, candesartan cilexetic (CDC), was identified as a novel neddylation inhibitor that ATP-competitively suppressing Nedd8-activating enzyme (NAE, E1) in mechanism, which inhibited the cullins neddylation superior than two representative non-covalent NAE inhibitors, M22 and mitoxantrone. Following with the findings such as apoptotic induction and tumor growth suppression in human lung cancer A549 *in vitro* and *in vivo*, CDC represents a potential anticancer lead compound with promising neddylation inhibitory activity.

Keyword: Neddylation, drug repurposing, candesartan cilexetic, NAE inhibitor.

1. Introduction

Protein neddylation is an important posttranslational modification that conjugates Nedd8 to substrates via a three-step enzymatic cascade reaction by E1 (NAE), E2s (Ubc12/UBE2M or UBE2F) and Nedd8 ligases (E3s, RBX1/ROC1, RBX2/ROC2, etc.) [1, 2]. In the process of neddylation, mature Nedd8 is first adenylated and then activated via binding NAE in an Mg^{2+}/ATP dependent manner to form NAE-Nedd8 [3]. Subsequently, activated Nedd8 is transferred to one of two E2s through a transthiolation reaction and forms the E2-Nedd8 [4]. Nedd8 is ultimately transferred from E2-Nedd8 to the specific substrates in the presence of E3s (Figure 1A). The best-known substrates of neddylation are members of the cullin family, which are involved in the assembly of cullin-RING E3 ubiquitin ligases (CRLs). CRLs act as a large family of ubiquitin ligases that target cellular protein substrates for proteasomal degradation [5, 6]. The inhibition of the neddylation pathway inactivates CRLs and decreases the protein levels of ubiquitination and subsequent degradation of substrates which are regulated by CRLs substrates, such as Wee1 [7], p27 [8], and Nrf2 [9], thereby leading to the suppression of the tumor progression. Besides CRL substrates, neddylation also regulates several non-cullin substrates, such as MDM2, p53, and VHL [10] (Fig 1A). In recent years, overactivated neddylation was observed in various types of human cancers and positively correlated with poor prognosis [11-15]. Thus, the inhibition of neddylation pathway has been developed as an attractive anticancer strategy recently [16-18].

NAE has emerged as a novel target in the neddylation pathway for drug discovery.

MLN4924 is a neddylation inhibitor that suppresses neddylation modification by covalently binding the adenosine triphosphate (ATP) binding pocket of NAE, which has been advanced into phase II/III clinical trials (Fig S1) [19, 20]. However, recent preclinical studies identified the resistance to MLN4924 for the heterozygous mutations nearby its covalently binding site with NAE, highlighting the necessity to develop other neddylation inhibitors [21, 22]. Therefore, Chung-Hang Leung and other researchers discovered a series of noncovalent NAE inhibitors by utilizing the structure-based virtual screening of compound libraries, including natural product 6, 6''-biapigenin, metal complex 1, M22, mitoxantron, 7g, LP0040 and deoxyvasicinone derivatives (Fig S1) [23-30]. Notably, the core skeleton of 7g, mitoxantron, LP0040 and deoxyvasicinone derivatives are similar to MLN4924, suggesting that these benzohetercyclic may be an essential group for maintaining the neddylation inhibitory effects of these inhibitors. Furthermore, long and flexible side links with amide or amino groups increased the affinities of compounds and NAE. In addition to NAE inhibitors, several E3 inhibitors were obtained by the high throughput screening (HTS) [31-35]. However, a majority of currently reported neddylation inhibitors lack enough druggability-related evaluations or structural potentiality, and accompany with seldom exploring the inhibitory effects of cullins neddylation comprehensively.

Utilizing new approaches and conceptions to discover novel therapeutic regimes and potential lead compounds has gradually become a trend in the field of drug research and development (R&D) [24, 36-38]. Drug repurposing is the re-exploration of clinically approved drugs and compounds for one indication being applied to new

and different diseases, which can significantly reduce cost and save time during the R&D of new drugs [39-41]. In this study, drug repurposing was performed for screening potential neddylation inhibitors, while the cullin1 neddylation level as the important phenotype was explored by an improved enzymatic assay in this screen. With a lab in-house “Old drug bank”, 1331 approved drugs were firstly screened *in vitro* with this assay, and investigated whether some agents could suppress the growth of human tumor cell with the neddylation inhibition. An antihypertensive agent candesartan cilexetil (CDC) presented superior inhibitory activity on cullin1 neddylation and structurally modificational potentiality than other hit compounds after two rounds of screening. Further experiments revealed that CDC was able to block cullins neddylation by inhibiting the NAE activity in enzymatic and cellular assays, which also displayed potent anticancer activity *in vitro* and *in vivo*. These evidences indicated that CDC is suitable for further structural modification as a lead compound. In addition, CDC as a benzimidazole-derived neddylation inhibitor is also similar to some reported inhibitors in the chemical structure [19-22]. This finding may afford us more rational design about novel derivatives in the following-up studies

2. Materials and Methods

2.1. Cell culture and reagents

Human lung cancer cell lines A549, EKVX, H1299, Human liver cancer cell lines HepG2, Huh7, Human breast cancer cell lines T-47D, MCF-7, Human gastric cancer cell lines MKN45, MGC803, PLC were obtained from the American Type Culture Collection (Manassas, VA) and passaged five to six times before use. Cells were

cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT), containing 10% fetal bovine serum (Biochrom AG, Berlin, Germany) and 100 units ml^{-1} penicillin/streptomycin solution and maintained in a humidified atmosphere of 5% CO_2 at 37°C (standard culture conditions). MLN4924, CDC and other drugs were dissolved in dimethyl sulfoxide (DMSO) and kept at -20°C for *in vitro* studies. MLN4924 and CDC were dissolved respectively in 5% 2-hydroxypropyl-β-cyclodextrin and 5% castor oil (Macklin Reagent, Shanghai, China) *in vivo* study. The solution of either MLN4924 or CDC was freshly made when was used.

2.2. Enzyme-based neddylation activity assay

The protein Nedd8, NAE, Ubc12, Rbx1/Cullin1^{CTD} were prepared and gifted by Prof. Jin Huang, Shanghai Jiao Tong University School of Medicine, China. The similar protocol was described [42] and applied by Prof. Yi Sun, Zhejiang University School of Medicine, China. In respect of drug screen, 1μL UBA3-NAE (final concentration, 0.025μM), 1μL Nedd8 (final concentration, 10 μM), 1μL Ubc12 (final concentration, 1μM), 1 μL RBX1/cullin1^{CTD} (final concentration, 1μM), 1μL Tris-HCl (1M, pH=7.4; final concentration, 50 mM), 1μL $MgCl_2$ (0.1M), 1μL DTT (10mM), 0.2μL 0.1mg/mL BSA, 2.8μL ddH_2O, were added into in each well of 96-well plates, followed with 2.0μL water solution of MLN4924 (final concentration, 10μM) or test drugs (final concentration, 50μM). 4.0μL (final concentration, 20μM) ATP was added to reaction after the mixture was incubated for 10min at room temperature. The mixture was incubated at 37°C for 30min. The reaction was quenched by 10% loading

buffer, and heated for 5 min at 95°C by a real-time PCR. Protein samples were electrophoresed under non-reducing conditions on a 10~15% SDS-PAGE gel, and cullin1-Nedd8 levels were determined by immunoblotting analysis. To determine the neddylation inhibitory activities of agent CDC, various concentration CDC groups (final concentration, 6.125μM, 12.5μM, 25μM, 50μM, and 100μM) were used. For the ATP-competitive assay, CDC (final concentration, 50μM) and ATP (final concentration, 0μM, 10μM, 100μM, 500μM, and 1000μM) were used, which all other conditions remained the same.

2.3. Cell-based neddylation activity assay

A549 cells were exposed to the indicated concentrations of MLN4924 (10μM) or CDC (25μM, 50μM, 75μM, 100μM, or 50μM, 60μM, 70μM, 80μM, 90μM, 100μM, or 50μM, 75μM, 100μM, 125μM) or 0.1% (v/v) DMSO for 6h or 12h, respectively. Cells were washed three times with ice-cold PBS, resuspended in RIPA lysis buffer, and incubated on ice for 30min. Cell debris was removed by centrifugation at 15,000rpm for 10min at 4°C. The protein concentration of the supernatant was determined with Thermo Fisher protein assay dye reagent (Thermo Fisher). Equal protein amounts were electrophoresed under non-reducing (cullin1-Nedd8, cullin2-Nedd8, cullin3-Nedd8, cullin4a-Nedd8, and cullin5-Nedd8) or reducing (Wee1, p27 or Clever-Parp) conditions on SDS-PAGEs and subjected to western blot analysis.

2.4. Cell proliferation and cell clonogenic assays

For evaluating the proliferation of cultured cells, cells seeded (A549, EKVX,

H1299, HepG2, Huh7, T-47D, MCF-7, MKN45, MGC803, and PLC) in 96-well plates with 2500 cells per well, in triplicate, and cultured overnight were treated with MLN4924 or CDC for 48 hours, followed by cell counting kit-8 (CCK-8) assay. For clonogenic assays, A549 cell were seeded in six-well plates (150 cells per well) in triplicate, and were treated with MLN4924 (0.1μM, 0.33μM, and 1μM) or CDC (5μM, 10μM, 15μM, and 20μM) and cultured for 10 days. Colonies were considered as 50 cells or more and counted. The representative results of three independent experiments with similar trend were presented.

2.5. Cell cycle and cell apoptosis assays

Cells treated with DMSO or various concentrations of agent CDC (25μM, 50μM, 75μM) were harvested and fixed in 90% ethanol at - 20°C for 24h, then centrifuged at 15,00rpm for 10min at 4°C in tubes. The residue in each tube was washed three times with ice-cold PBS, and stained with 0.5mL propidium iodide solution (36μg/mL; Sigma, St. Louis, MO) contained RNase A (10μg/mL; Sigma) at 37 °C for 30 minutes, and analyzed for cell-cycle profile with Flowjo. Representative results of three independent experiments with similar trends are presented.

Apoptosis was evaluated using FITC Annexin V apoptosis detection kit. A549 cells were seeded at 2.5×10^5 cells per well in 6-well plates and allowed to attach overnight. Cells were treated with increasing concentrations of CDC (25μM, 50μM, 75μM) for 48h. Cells were dissociated using trypsin, washed three times with ice-cold PBS, and resuspended in 100μL 1×binding buffer. The addition of 5μL FITC Annexin V staining solution and 5μL PI staining solution were added. After incubation at room

temperature in the dark for 15min, another 400μL 1×binding buffer was added. Stained cells were analyzed immediately by fluorescence-activated cell sorting (FACS).

2.6. Molecular modeling

The X-ray structure of NAE was downloaded from the Protein Data Bank (PDB ID: 3GZN). Crystallographic water, ligands and alternate conformations were removing by using the protein preparation protocol in Autodock 4.0. The docking procedure was performed by employing DOCK program in Autodock 4.0, and the structural image was obtained using PyMOL software.

2.7. Tumor xenograft growth inhibition

Female nude mice were subcutaneously injected with 2×10^6 A549 cells in 100 μL PBS according to protocols of tumor transplant research. After tumor induction (about 7 days), nude mice were divided into five groups randomly, including mock group (10% 2-hydroxypropyl-β-cyclodextrin/water solution), positive group (MLN4924-60 mg/kg), and the other three were for various treatments of CDC, respectively. Compounds were administrated by intraperitoneal injection (60 mg/kg vs 30 mg/kg). All groups were treated with one dose per day. Body weights and tumor volumes were measured every 2 days. On the 32th day after inoculation, all the mice were sacrificed. Tumor volumes and mass were measured for each group.

3. Results and Discussion

3.1. Discovery of optimal neddylation inhibitor CDC

Cullin1-regulated CRL (CRL1) emerges as the most studied cullins-associated

complex in neddylation [43-45]. In order to rationally evaluate the neddylation inhibitory activities of the approved drugs, an enzyme-based assay that simulated the process of cullin1 neddylation *in vivo* was set up in this study. The assay was operated with ATP under the cascade reaction of protein Nedd8-E1 (NAE)-E2 (Ubc12)-E3 (RBX1)-cullin1 pathway (Figure 1B). Taking advantage of the assay, we primarily screened 1331 FDA-approved drugs and explored their neddylation inhibitory effects (Figure 1C). Theoretically, Nedd8 in control groups with ATP could conjugate the substrate cullin1, thereby forming the cullin1-Nedd8 adduction, whereas the groups without ATP failed to form cullin1-Nedd8 adduction (Figure S2). Similar to the groups without ATP, MLN4924 groups competitively suppressed the formation of cullin1 neddylation in the presence of ATP (Figure S2, **WB-01**). If the cullin1-Nedd8 conjugation of the drug-treated groups decreased similar to that of MLN4924, these data would suggest these drugs are neddylation inhibitors. In this assay, 106 drugs that presented either partially accumulating of cullin1 or deceasing of cullin1-Nedd8 adduction were identified as hit compounds and selected for reinspection. (Figure S3).

In the secondary hit compounds screening, five hit drugs displayed superior neddylation inhibitory activities than the others, including CDC (**01**, MT04D0301), Trifluoperazine (**02**, MT05C1901), Bismuth Potassium Citrate (**03**, MT08A1201), Cetylpyridinium Chloride (**04**, MT20B0301), and Procyanidin (**05**, MT24A0201) (Figure 1D). In this study, we first investigated the cullin1-Nedd8 adduction inhibition of those five hit compounds by a cell-based assay for removing the pan-assay interference compounds (PAINS) in the enzymatic assay. In general, **CDC** presented

significant neddylation inhibitory activity at the concentration of 100μM comparable to MLN4924, whereas hit compounds **02**, **03**, and **04** failed to display efficacy (Figure S4). Meanwhile, **05** not only showed inferior neddylation inhibitory activities than CDC, but seriously violated the Lipinski's rule of five in the respect of structure as well [46, 47]. Thus, drug CDC was selected as the best lead compound to explore the mechanism of neddylation inhibition.

3.2. CDC suppresses neddylation pathway via inhibiting NAE

To further explore the efficacy and interactive mechanism of CDC, a dose-dependent experiment with various concentration of CDC was firstly performed for measuring the level of cullin1-Nedd8 adduction. Meanwhile, since NAE activates Nedd8 and transfers it to Ubc12 in the process of neddylation, the level of Ubc12-Nedd8 conjugation was assessed in parallel for preliminary determining target enzyme of CDC. The results showed that the formation of cullin1-Nedd8 adduction was significantly inhibited by CDC at the concentration of 25~50μM, while the formation of Ubc12-Nedd8 adduction was completely inhibited at 50μM as well (Figure 2A), suggesting that CDC inhibits the enzyme in the upstream of neddylation (NAE or Ubc12). ATP-binding pocket of NAE emerged as an excellent drug target with distinct mechanism in neddylation has been utilized for the discovery of neddylation inhibitors, such as MLN4924, M22, mitoxantrone. Thus, an ATP concentration-dependent assay was conducted to determine whether CDC was an ATP-competitive inhibitor. The results showed that the inhibitory activities of CDC on cullin1-Nedd8 and Ubc12-Nedd8 were reduced with increasing concentrations of ATP

(Figure 2B).

Furthermore, in order to obtain enzyme inhibition properties of CDC, a kinetic analysis on the inhibition was performed using Lineweaver-Burk representation analysis. The Lineweaver-Burk plot showed that the Vmax values were regardless of concentration of CDC while the Km values were elevated with increasing concentrations of CDC (Figure S5), demonstrating that CDC represents an ATP-competitive inhibitor to suppress the neddylation. Meanwhile, an additional kinetics binding assay was addressed for exploring the binding affinity of CDC with NAE by the application of surface plasmon resonance (SPR) technology. The SPR results showed CDC displayed moderate binding effect (K_D=195μM) with NAE (Figure S6). Consequently, these results suggested that CDC acts as an ATP-competitive neddylation inhibitor by targeting the enzyme NAE.

We have previously shown the overactivated neddylation-CRLs axis amplified the lung cancer development [13]. Thus, we chose human A549 lung cancer cells to explore the efficacy against the neddylation modification, and then identified the level of global protein neddylation using a specific Nedd8 antibody to investigate the efficacy of CDC against the neddylation pathway (Figure 2C). In contrast to the control group, the treatment groups could diminish the level of global protein neddylation, even comparable to MLN4924. Cullin family members act as the best-known substrates of neddylation that involved in the assembly of CRLs are modulated by Ubc12 and UBE2F. In cullin family members, individual cullin1, 2, 3, 4a or 4b is catalyzed by the Ubc12 neddylation, while cullin5 is catalyzed by the

UBE2F neddylation [48, 49]. In the present study, those characteristics were utilized for evaluating the neddylation inhibitory ability and identifying target enzyme of CDC by comprehensively exploring the levels of cullins-Nedd8 adduction (Figure 2D). In general, CDC represented an efficient neddylation inhibitor for suppressing the whole cullins neddylation significantly at 50μM. On the other hand, the neddylation inhibition resulted in a corresponding decrease in the abundance of cullins-Nedd8 with the dosage increase of CDC, indicating that the whole neddylation pathway was blocked by CDC. Meanwhile, the degradations of two representative CRLs substrates Wee1 and p27 were decreased after incubating with CDC in this assay (Figure 2E), validating that CDC stabilized CRLs substrates by suppressing neddylation-CRLs pathway.

Nedd8 activating enzyme NAE in neddylation pathway is closely related to some enzymes in ubiquitination or sumoylation [19], suggesting that CDC may also block these pathways. Herein, Sumo-conjugating enzyme Ubc9, Ub-conjugating enzyme UbcH10, Sumo1 and ubiquitin antibodies were introduced for investigating the effects of CDC on ubiquitination and sumoylation by immunoblotting (Figure S7). The results showed that the formation of either Ubc9-Sumo or Ubc10-Ub adduct were almost not suppressed at different concentrations of CDC. Furthermore, the levels of Sumo1- and Ub-modified proteins also had no significant decrease in the presence of CDC, suggesting that CDC selectively blocks the neddylation pathway. Small molecule M22 as a known noncovalent NAE inhibitor that displays the significant antitumor activities *in vitro* and *in vivo* was suitable for comparing the inhibition of

CDC [25]. In this study, we synthesized compound M22 (Figure S8) and compared its cullin1-Nedd8 and global Nedd8 modification inhibitory effects with those of CDC. Despite M22 had significant inhibitory effect against Nedd8-associated conjugation at the range of 60~80μM in correspondence to literature reports, the cullin1-Nedd8 and global neddylation inhibition of M22 was inferior to those of CDC (Figure S9A). Similarly, another known NAE inhibitor mitoxantrone (an antitumor drug) was also used in our study to compare with CDC [26]. The results showed that mitoxantrone had no effect on cullin1 neddylation at the concentration of 50μM but partly inhibited the formation of cullin1-Nedd8 at 75μM, which was inferior to CDC as well (Figure S9B).

3.3. The effect of neddylation inhibitor CDC on anticancer activity *in vitro*

Considering that blocking the neddylation pathway can effectively suppress tumor progression [11-15], we examined the antiproliferation activities of CDC against A549 cell by CCK-8 cell viability assay (Figure 3A). The results displayed that CDC could inhibit A549 cell proliferation in the micromolar range (IC_{50}=63.93±4.18μM). To further investigate the anti-cell survival effects of CDC, colony formation assay was performed as a side-by-side assay (Figure 3B, C). Similarly, the results demonstrated CDC inhibited the clonogenic survival in a dose-dependent manner.

To further explore whether growth inhibition suppressed by CDC was associated with cell cycle, DNA content of cell nuclei was detected by flow cytometry, as shown in Figure 3D. In general, phases G1, S, G2 had no significant difference in various concentrations of CDC-treated groups in 24h, whereas the cell population in Sub-G1

phase increased 0.89% to 30.45%, suggesting that CDC induced cancer cell apoptosis. Moreover, Annexin-V/PI double staining assay was further performed for evaluating the capacity of CDC to induce apoptosis (Figure 3E). After treatment with various concentrations (25, 50, or 75μM, 24h) of CDC, the total numbers of early and late apoptotic cells were 9.6%, 25.2%, and 50.9%, respectively. In addition, we found that cleaved-PARP (Poly ADP ribose polymerase, cleaved by caspase-3), as another classical apoptosis marker, was accumulated after treatment with the increasing concentration of CDC (Figure 3F). Taken together, these results validated that the treatment of CDC induced cell death through apoptosis in the A549 cells.

To evaluate the capacity of CDC against other cell lines, we tested additional nine cancer cell lines using the CCK-8 assay. These cell lines included EKVX (lung cancer), H1299 (lung cancer), HepG2 (liver cancer), Huh7 (liver cancer), T-47D (breast cancer), MCF-7 (breast cancer), MKN45 (gastric cancer), MGC803 (gastric cancer), and PLC (gastric cancer), as shown in Table 1. We observed that CDC decreased the proliferation of various types of cell lines in the micromolar range. In addition, two normal cell lines BEAS-2B (Normal lung epithelial cell) and 16HBE (Normal bronchial epithelial cell) were utilized for exploring the selectivity of CDC (Table 1). In general, neddylation inhibitor CDC presented minor proliferation inhibitory activities against these two normal cell lines (IC_{50} = 89.94 ± 5.16μM and IC_{50} > 100μM, respectively), along with the overactivation of target neddylation pathway in various cancer tissues in vivo as described above [11, 12], suggesting that CDC has a certain selective effect in suppressing cancer cell lines.

3.4. The neddylation inhibitory activity of CDC originates from its unique structure

Molecular modeling studies were performed for investigating the binding mode between CDC and NAE by Autodock 4.0. As shown in Figure 4A, the binding mode of CDC in the NAE complex was similar to that of MLN4924 in general, but its binding conformation had some difference with MLN4924 (Figure 4B, C). In comparison with MLN4924 (PDB ID: 3GZN), CDC was estimated to form various interactions in the binding pocket, including two H-bonds to both the side chain guanidine group of Arg22 (3.1Å) and carboxylate group of Asp167 (1.9Å) through its tetrazolium substituent. Meanwhile, CDC had the same H-bond to the side chain amide group of Gln149 (2.3Å) through its tetrazolium cilexetil substituent as that of MLN4924. Unlike MLN4924, CDC lacked the sulphamate group to form the covalent adduct with NAE. These molecular interactions suggested that CDC had high affinity with NAE comparable to MLN4924.

CDC is a well-known prodrug of candesartan that owns the moderate angiotensin II type 1 receptor (A2T1R) antagonistic activity, which transfers into potent candesartan by oral administration. In this study, we could not preclude the possibility that CDC would suppress the neddylation through its A2T1R antagonistic activity. Thus, other nine FDA-approved A2T1R antagonists, including losartan, irbesartan, azilsartan, olmesartan medoxomil, eprosartan, telmisartan, valsartan, candesartan, and valsartan were explored for neddylation inhibitory activities in A549 cell line (Figure 4D). The results showed that formations of cullin1-Nedd8 failed to be blocked after the treatment of these antagonists in contrast to that of CDC. Notably, candesartan, a

metabolic product of CDC, displayed no anti-neddylation activity, indicating that the inhibition effect of CDC is related to its unique structure and irrelevant to A2T1R antagonistic effect.

3.5. Neddylation inhibitor CDC suppresses the growth of A549 xenograft tumors in mice

To evaluate the antitumor activity of CDC *in vivo*, we administered CDC to nude mice bearing human tumor cell xenografts and monitored tumor growth rate. Tumors were established after 8 days after subcutaneous inoculation of A549 lung cancer cells (the tumor volume is at 70~100mm^3), tumor bearing mice were then randomly allocated into four groups: mock group, MLN4924-treated group as positive control (60mg/kg qd) and CDC-treated groups (60mg/kg qd and 30mg/kg qd) with six mice per group. The indicated agents were administrated by intraperitoneal injection. As shown in Figure 5A, B, in contrast to mock group, all treatment groups significantly decreased tumor volume and tumor weight after 22 days. Furthermore, CDC displayed the dose-dependent activities through comparative analyses with those CDC treatment groups (Figure 5A). To our surprise, although the MLN4924 group presented superior antitumor activity in the assay, CDC treated with 60mg/kg group had no significant difference with MLN4924 *in vivo*. Compared with either Mock or MLN4924 groups, CDC-treated with 60 or 30mg/kg group did not affect the body weight (Figure 5B). In addition, the tumor weights and sizes were measured and the two CDC-treated groups had a decrease in tumor growth compared to the Mock group (Figure 5C, D). In particular, CDC-treated with 60 mg/kg group decreased the tumor

weight comparable with MLN4924 group. Taken together, these data indicated that CDC was efficacious in suppressing tumor growth *in vivo* with no observable toxicity.

4. Conclusion

One major challenge in developing neddylation inhibitors as therapeutic drugs is lack of enough druggability studies and the costs of research and development. The advantage of drug repurposing is cost and time effectiveness as absorption, distribution, metabolism, excretion, and basic toxicity are already well established and can be immediately taken to phase II/III clinical trials [39-41]. In this study, we screened 1331 approved drugs from our in house “Old drug bank” to discover CDC as a potential neddylation inhibitor based on a new enzyme-based assay with evaluating the cullin1-Nedd8 inhibition. The findings from this study demonstrated the practicability of this screening strategy for the discovery of novel neddylation inhibitors.

The additional enzyme-based assays revealed that CDC not only significantly inhibited neddylation pathway by evaluating the formations of cullin1-Nedd8 and Ubc12-Nedd8 adductions, but ATP-competitively suppressed the neddylation modification as well. As for cellular-based assays, CDC could significantly suppress various cullins neddylation pathway in cancer cells, comparable to its performance in enzymatic assay. Further study with investigating the inhibitions of other structurally similar A2T1R antagonists validated that the neddylation inhibition of CDC originated from its unique structure, not related to A2T1R antagonistic activity. A molecular modeling verified the high affinity of CDC with NAE. In addition, CDC

inhibited the cullins neddylation more significant than two representative NAE inhibitors M22 and mitoxantrone. Taken together, CDC represents a novel neddylation inhibitor for suppressing the enzyme NAE.

Furthermore, anticancer experiments *in vitro* showed that CDC inhibited cancer cell growth and survival in the micromolar range. Additional cell cycle and Annexin-V/PI double staining assays displayed that CDC induced apoptosis in the A549 cancer cell. Antitumor activity *in vivo* indicated CDC significantly inhibited the growth of A549 xenograft tumors in mice. These findings demonstrated that CDC presents the passable efficacy on suppressing the tumor growth *in vitro* and *in vivo*. However, the neddylation and anticancer inhibitory activities of CDC are inferior to those of clinical agent MLN4924. Meanwhile, the defects of structurally easy hydrolysis by oral administration and potential A2T1R-related toxicity also limit CDC to develop a novel cancer therapeutic drug. Thus, subsequent studies will focus on the structural modification by medicinal chemistry to overcome those weaknesses. Especially, based on the results of docking model, we will chemically modify the hydrolyzable cilexetil group in the structure of CDC for elevating the neddylation and anticancer inhibitory activities and *in vivo* stability. In summary, CDC significantly inhibits the neddylation modification by targeting enzyme NAE, and represents a promising lead compound for the development of new anticancer drugs.

ASSOCIATED CONTENT

Supporting Information

Current reported neddylation inhibitors; The list of “Old Drug Bank”; Primary

approved drugs screen blocks the cullin1-Nedd8 pathway at the concentrate of 50μM; Secondary hit compounds screen blocks the cullin1-Nedd8 pathway at the concentrate of 50μM; The synthetic route of M22 and the related spectrum; The preliminary neddylation inhibitory activities of hits compounds in human lung cancer cell A549; Lineweaver-Burk representation of the kinetics assay; The kinetics binding assay by the surface plasmon resonance (SPR) technology; Various concentrations of M22 and mitoxantrone blocked the cullin1-Nedd8 pathway and Nedd8-mediated modification in A549 cell.This material is available free of charge via the Internet.

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Author Contributions

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Notes

The authors declare no competing financial interests.

Abbreviations

CRLs, cullin-RING ligases; Nedd8, neuronal precursor cell-expressed developmentally down-regulated protein 8; NAE, Nedd8-activating enzyme E1; ATP, adenosine triphosphate; A2T1R, Angiotensin II type 1 receptor; intraperitoneal injection, I.P.

Ethics approval and consent to participate

All experimental protocols were approved by the Animal Experimental Ethics

Committee of Longhua Hospital of Shanghai University of Traditional Chinese Medicine.

Conflicts of interest statement

The authors agree with the content of this manuscript and declare there are no competing interests about this study.

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Figure legends

Figure 1. (A) The process of protein modification by neddylation and reported neddylation inhibitors. (B) The screening strategy that exploration the inhibition of approved drugs on the Nedd8-NAE-Ubc12-RBX1-cullin1 pathway was conducted analyzed by immunoblotting. (C) The process of discovery of neddylation inhibitors from “Old Drug Bank”. (D) Hit compounds and lead compound neddylation inhibitor CDC.

Figure 2. (A) Various concentrations of CDC inhibited both cullin1-Nedd8 and Ubc12-Nedd8 formation in the enzyme-based assay. (B) CDC inhibited both cullin1-Nedd8 and Ubc12-Nedd8 formation with the addition of various concentrations of ATP in an enzyme-based assay. (C) CDC inhibited the global Nedd8 modification in A549 cell. (D) CDC blocked the cullins-Nedd8 pathway in A549 cells. (E) CDC suppressed degradation of CRLs substrates in A549 cells.

Figure 3. The antitumor activity of agent CDC on A549 lung cell *in vitro*. (A) Survival curve of CDC against A549 (n=3). (B, C) A549 was treated with either MLN4924 (0.1, 0.33, and 1μM) or CDC (5, 10, 15, and 20μM) at indicated doses to determine its therapeutic efficacy on clonogenic survival (C, ***P < 0.001). (D) CDC induced Sub-G1 arrest in A549 cancer cells. A549 cells were incubated with DMSO and various concentrations of CDC (25, 50, and 75μM) for 24 h. Cells were harvested and stained with PI and then analyzed by flow cytometry (G1 in red, S in green, G2 in blue and Sub-G1 in white). The percentages of cells in different phases of the cell cycle were analyzed by FlowJo. Histograms displayed the percentage of cell cycle distribution. (E) Evaluation of apoptosis on A549 cell line by Annexin V/PI staining

and flow cytometry detecting. 0.1% DMSO treated A549 cell in 48h; CDC treated A549 cell in 48h at the concentration of 25μM; CDC treated A549 cell in 48h at the concentration of 50μM; CDC treated A549 cell in 48h at the concentration of 75μM. Statistical significance was determined by the Mann-Whitney test (two-tailed): ***$p < 0.001$, n.s. indicates no significant difference. (F) CDC suppressed degradation of apoptotic protein cleaved-parp in A549 cells.

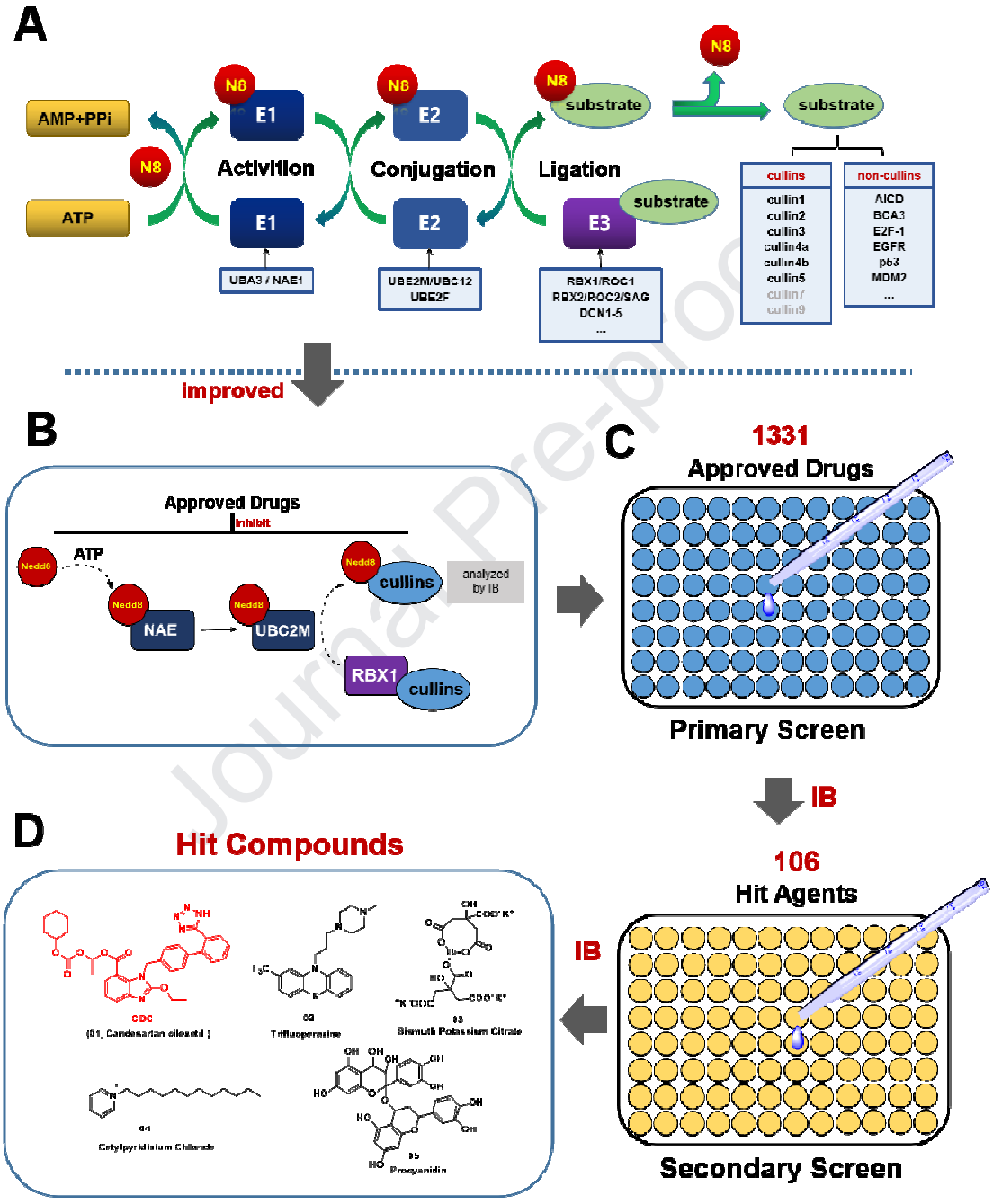
Table 1. IC_{50} values of CDC or MLN4924 treated various cancer cell lines or normal lung cell lines after 48h (μM, n=3).

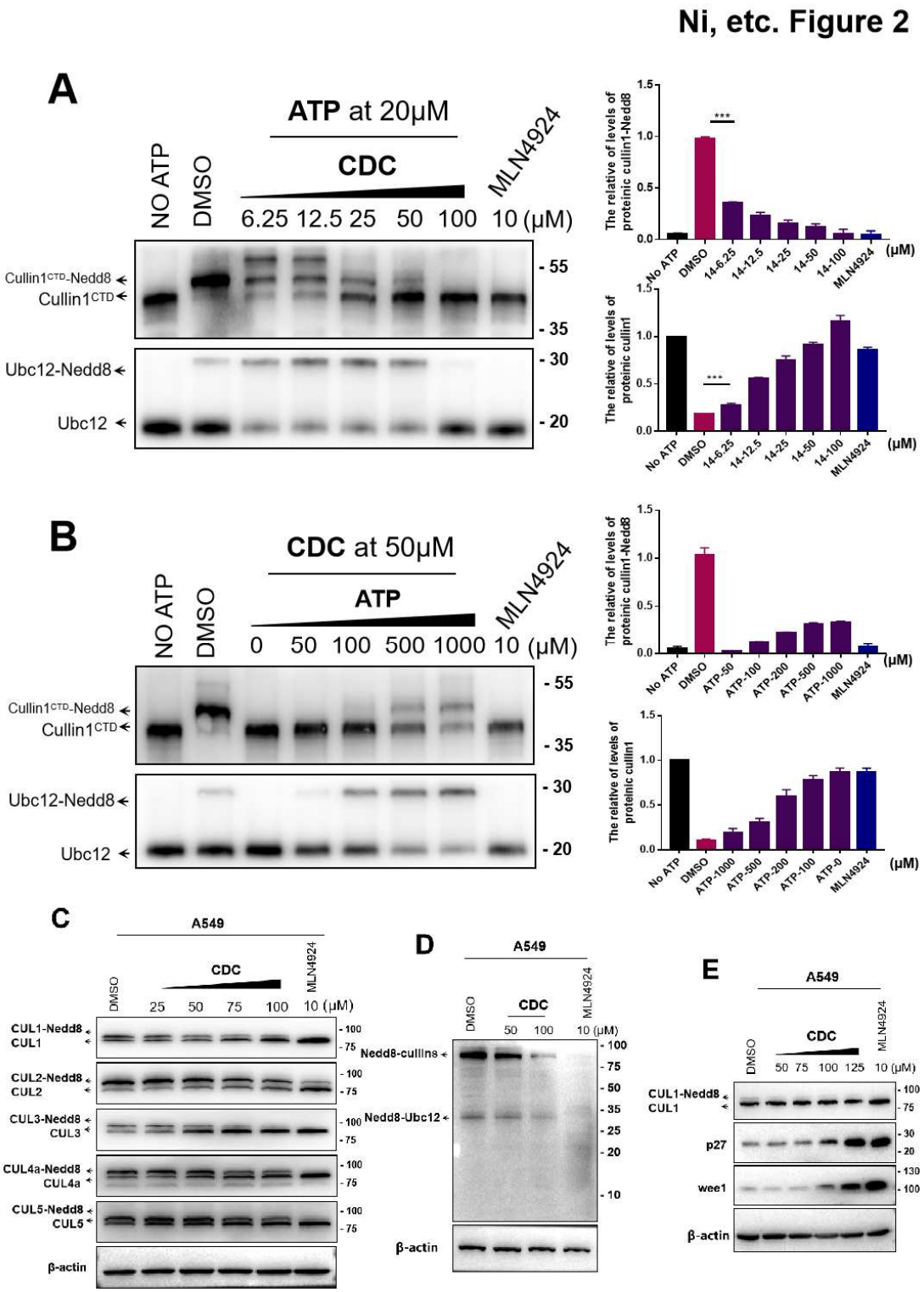
Figure 4. Molecular modeling results. (A) Merging between top-ranked CDC pose (shown in blue) and MLN4924 conformation (shown in purple) from NAE crystal (PDB ID: 3GZN). (B, C) Low-energy binding conformations of CDC (shown in blue) bound to NAE heterodimer generated by virtual ligand docking. The binding pocket of NAE is represented as a gray ribbon form. Amino-acid residues and small moleculars are depicted as a stick model showing carbon (gray, blue, or purple), hydrogen (white), oxygen (red), nitrogen (blue), and sulfur (yellow) atoms. H-bonds are indicated as black lines. (D) Further investigating the inhibitory effects of A2T1R antagonists on the neddyaltion pathway.

Figure 5. CDC inhibited A549 xenograft growth in nude mice (n=6). After intraperitoneally administering vehicle (black), MLN4924 (60 mg/kg, red), CDC (60 mg/kg, orange), CDC (30 mg/kg, blue) for three weeks, the mice were sacrificed, and the tumors were weighed. (A) Tumor volume changed during treatment; (B) Body weight changed of mice during treatment. (C) The images of tumors from mice at 21

days after initiation of treatment; (D) The tumorous weight of each group. Statistical significance was determined by the Student's t test (two-tailed): **p<0.01, ***p<0.001, n.s. indicates no significant difference.

Ni, etc. Figure 1




Ni, etc. Figure 2
A
ATP at 20μM
CDC
NO ATP
DMSO
6.25 12.5 25 50 100 10 (μM)
MLN4924
Cullin1CTD-Nedd8
Cullin1CTD
Ubc12-Nedd8
Ubc12
- 55
- 35
- 30
- 20
The relative of levels of proteinic cullin1-Nedd8
The relative of levels of proteinic cullin1
No ATP
DMSO
14-6.25
14-12.5
14-25
14-50
14-100
MLN4924
(μM)
B
CDC at 50μM
ATP
0 50 100 500 1000 10 (μM)
ATP-50
ATP-100
ATP-200
ATP-500
ATP-1000
ATP-0
C
A549
CDC
DMSO
25 50 75 100 10 (μM)
CUL1-Nedd8
CUL1
CUL2-Nedd8
CUL2
CUL3-Nedd8
CUL3
CUL4a-Nedd8
CUL4a
CUL5-Nedd8
CUL5
β-actin
D
Nedd8-cullins
Nedd8-Ubc12
E
50 75 100 125 10 (μM)
p27
wee1

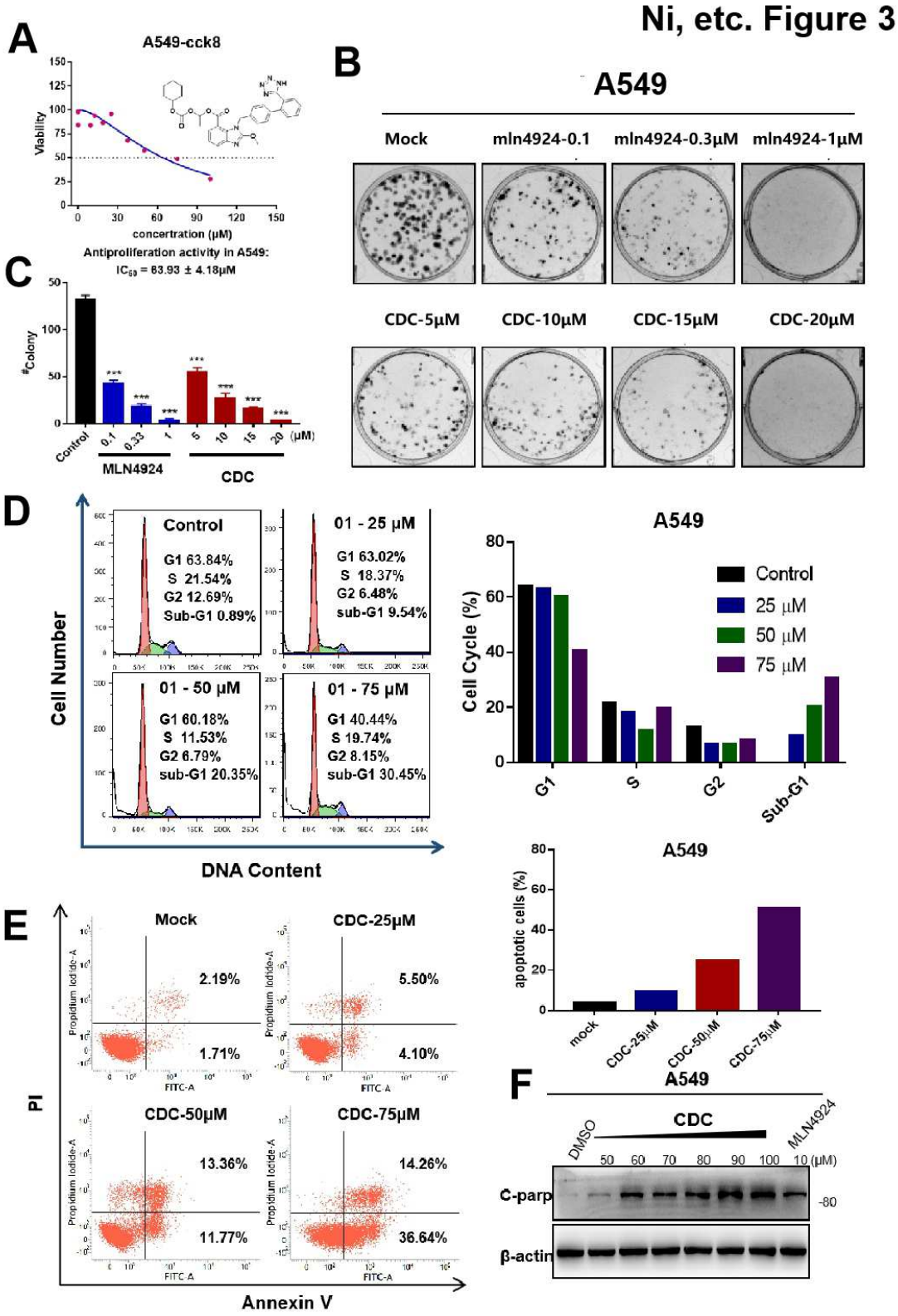
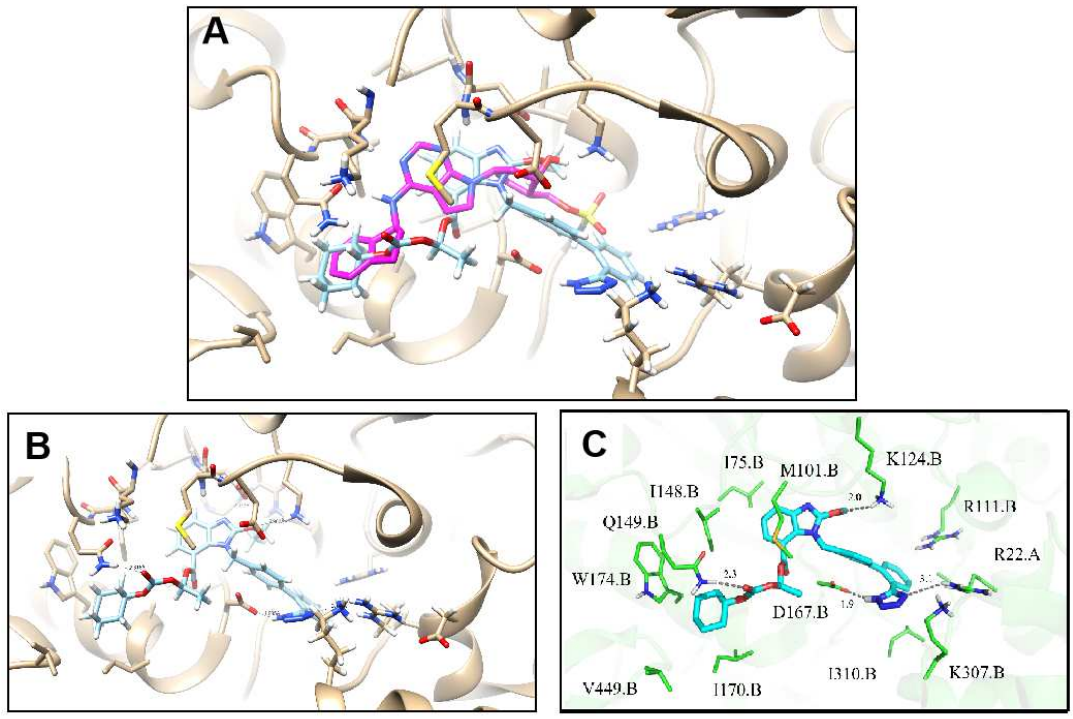

Ni, etc. Figure 3
A
A549-cck8
Viability
concertration (μM)
Antiproliferation activity in A549:
IC50 = 83.93 ± 4.18μM
B
A549
Mock
mln4924-0.1
mln4924-0.3μM
mln4924-1μM
CDC-5μM
CDC-10μM
CDC-15μM
CDC-20μM
C
#Colony
Control
MLN4924
CDC
D
Control
G1 63.84%
S 21.54%
G2 12.69%
Sub-G1 0.89%
01 - 25 μM
G1 63.02%
S 18.37%
G2 6.48%
sub-G1 9.54%
01 - 50 μM
G1 60.18%
S 11.53%
G2 6.79%
sub-G1 20.35%
01 - 75 μM
G1 40.44%
S 19.74%
G2 8.15%
sub-G1 30.45%
Cell Number
DNA Content
A549
Cell Cycle (%)
Control
25 μM
50 μM
75 μM
G1
S
G2
Sub-G1
E
Mock
2.19%
1.71%
CDC-25μM
5.50%
4.10%
CDC-50μM
13.36%
11.77%
CDC-75μM
14.26%
36.64%
PI
Annexin V
A549
apoptotic cells (%)
mock
CDC-25μM
CDC-50μM
CDC-75μM
F
A549
CDC
DMSO
MLN4924
50 60 70 80 90 100 10 (μM)
C-parp
-80
β-actin

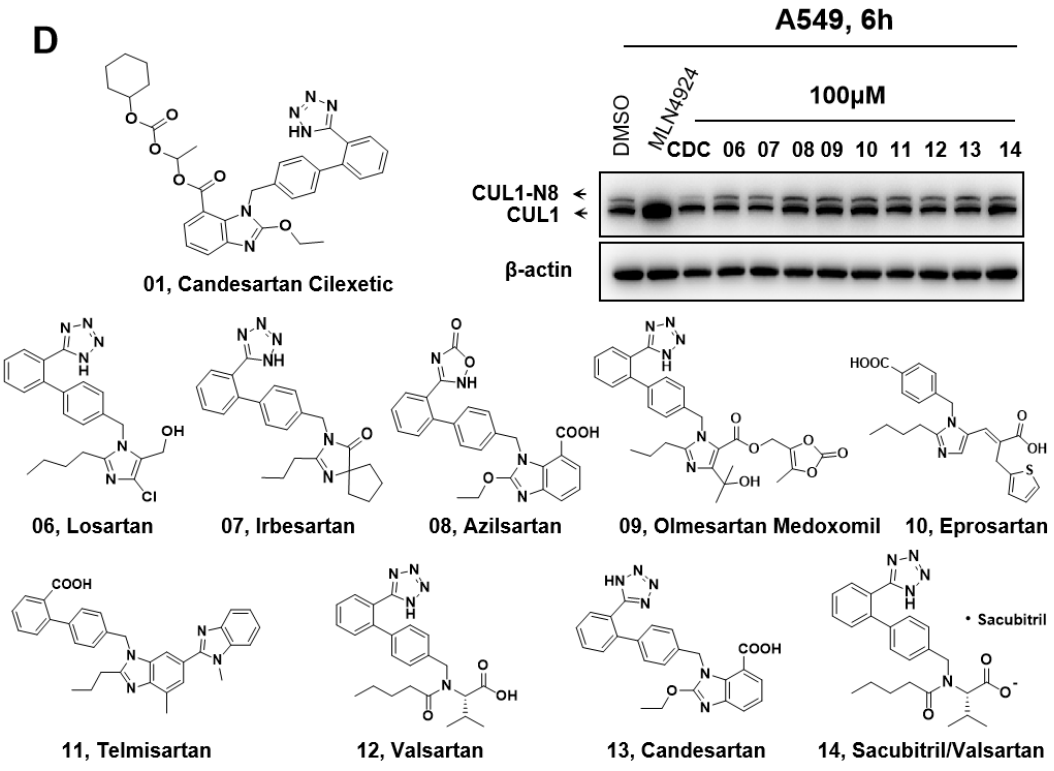
Table 1. IC_{50} values of CDC or MLN4924 treated various cancer cell lines or normal lung cell lines after 48h (μM, n=3).

Cell line	Tissue Type	CDC（μM）	Cell line	Tissue Type	CDC（μM）
EKVX	Lung cancer cell	> 100	**MKN45**	Gastric cancer cell	68.87 ± 2.22
H1299	Lung cancer cell	45.11 ± 2.17	**MGC803**	Gastric cancer cell	57.14 ± 3.36
HepG2	Liver cancer cell	> 100	**PLC**	Gastric cancer cell	60.21 ± 4.31
Huh7	Liver cancer cell	> 100	**BEAS-2B**	Normal lung epithelial cell	89.94±5.16
T-47D	Breast cancer cell	64.35 ± 6.41	**16HBE**	Normal bronchial epithelial cell	> 100
MCF - 7	Breast cancer cell	> 100			
MLN4924	IC_{50} > 10μM against various cancer cell lines after 48h IC_{50} at the range of 0.3-1.2μM against various cancer cell lines after 72~96h				

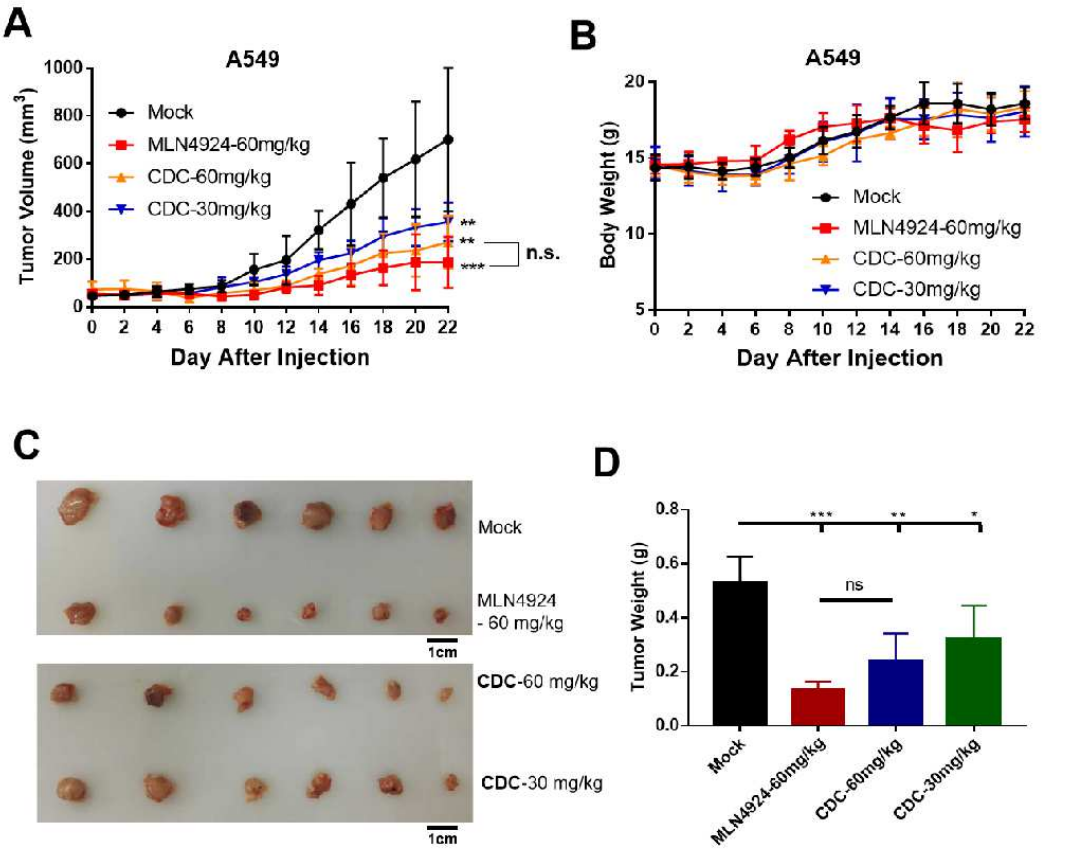
Ni, etc. Table 1.

Ni, etc. Figure 4





Ni, etc. Figure 5



1. An improved screening system was set up for exploring neddylation inhibitor.
2. 1331 approved drugs were investigated the inhibitory effects on the formations of cullin1-Nedd8 adduction via this screening assay.
3. CDC was identified as a novel NAE inhibitor for selectively suppressing the tumor growth by inducing the apoptotic.
4. Additional investigating of CDC analogs and molecular modeling validated that the neddylation inhibition of CDC originated from its unique structure.

Declaration of Interest Statement

This manuscript (title: **Discovery of candesartan cilexetic as a novel neddylation inhibitor for suppressing tumor growth** (manuscript ID: EJMECH-D-19-02331))has not been published or presented elsewhere in part or its entirety, and is not under consideration by another journal. There are no conflicts of interest to declare.