Journal Pre-proofs

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PII: S0960-894X(19)30756-5
DOI: https://doi.org/10.1016/j.bmcl.2019.126791
Reference: BMCL 126791

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 19 August 2019
Revised Date: 28 October 2019
Accepted Date: 29 October 2019

Please cite this article as: Song, J., Cui, X-X., Wu, B-W., Li, D., Wang, S-H., Shi, L., Zhu, T., Zhang, Y-B., Zhang, S-Y., Discovery of 1,2,4-triazine-based derivatives as novel neddylation inhibitors and anticancer activity studies against gastric cancer MGC-803 cells, *Bioorganic & Medicinal Chemistry Letters* (2019), doi: https://doi.org/10.1016/j.bmcl.2019.126791

Discovery of 1,2,4-triazine-based derivatives as novel neddylation inhibitors and anticancer activity studies against gastric cancer MGC-803 cells

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Abstract:

Neddylation modification is often over-expressed in a variety of human tumor cells. Therefore, targeting neddylation pathway may represent a potential approach to the treatment of human tumors. Herein, we describe the discovery of a hit scaffold from our in-house library and further structure-based optimizations. In this work, compound **V11** could block the neddylation and inhibit the activity of NAE (with an EC_{50} value of 3.56 μM), and a dose-dependent reduction of the Ubc12-NEDD8 conjugations was also observed. Molecular docking results suggest compound **V11** could bind tightly to NAE via hydrogen bonds and hydrophobic interactions. Compound **V11** showed the best antiproliferative ability with an IC_{50} value of 8.22 μM against gastric cancer MGC-803 cells. Further anticancer activity studies suggested that compound **V11** inhibited MGC-803 cell growth, caused a cell cycle arrestment at G2/M phase and induced apoptosis via extrinsic and intrinsic apoptosis pathways. All the findings suggest that 1,2,4-triazine scaffold might provide a novel scaffold for the further development of neddylation inhibitors and compound **V11** might be a potential neddylation inhibitor with anticancer activity.

Keywords: 1,2,4-Triazine; Neddylation; MGC-803; UBC12-NEDD8 conjugation;

Neddylation is a novel type of posttranslational modification, in which the Neural Precursor Cell-Expressed Developmentally Downregulated protein 8 (NEDD8) can be conjugated to substrate proteins in a process and regulates their functions[1-3]. In neddylation cascade, an E1 enzyme (NEDD8-activating enzyme (NAE; a heterodimer of NAE1 and UBA3 subunits)) activates NEDD8 and then transfers it to an E2 enzyme (NEDD8-conjugating E2 enzyme, also known as UBC12). Finally, the NEDD8 is conjugated to cullin proteins to activate the cullin-RING ubiquitin E3 ligases (CRLs) activity[4-6](**Fig.1**). Neddylation regulates a wide range of biological processes such as cell cycle, signal transduction and immune recognition[1, 7-9]. Neddylation modification is highly activated in many cancer cells and promotes cancer development such as gastric cancer[10], lung cancer[8], liver cancer[11]. Further studies suggest blocking the neddylation modification has anti-tumor effects[12-15]. Therefore, targeting neddylation pathway may represent a potential approach to the treatment of human cancers.

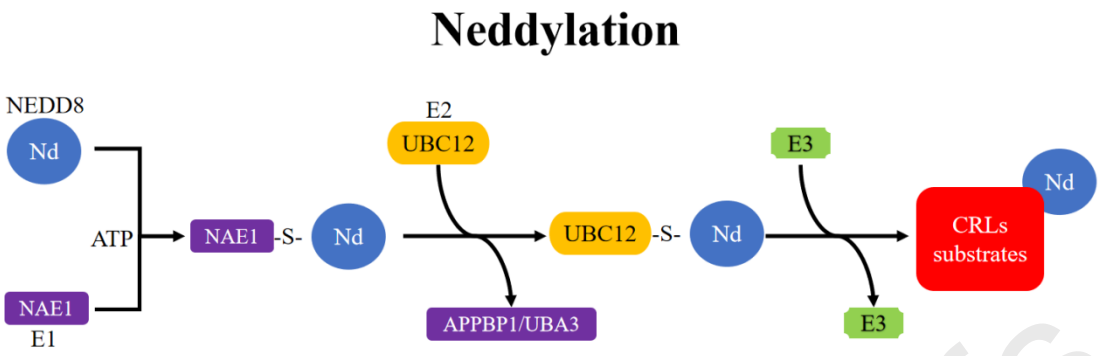


Fig. 1 The neddylation pathway

Targeting neddylation pathway has emerged as a new approach to the treatment of human cancers since the finding of NEDD8-activating enzyme (NAE) inhibitor MLN4924[15]. MLN4924 could covalently bind to NAE, block all CRLs neddylation and cause the accumulation of CRLs substrates[15, 16]. MLN4924 has entered phase II clinical trials at present and showed effective activity against both solid and hematological human cancers[17]. However, as results of its broad ablation of neddylation, MLN4924 has a series of toxicities[18]. Recently, some other neddylation inhibitors have been reported[15, 19-27]. Compound **1** was reported as a novel NAE inhibitor which inhibited NEDD8 protein and subsequently elevated UBC12 levels[20]. Compound **2** inhibited NAE/UAE activities, downregulated degradations of related substrates in AGS cells, and promoted apoptosis in low micromole concentrations[21]. Compound **3** inhibited cullins neddylation and the DCN1−UBE2M protein−protein interaction in TR-FRET binding assay(IC_{50} = 62 nM)[23](**Fig.1**). Compound **4** named **WS-383** reversibly blocked the DCN1-UBC12 interaction (IC_{50} = 11 nM), exhibited cellular target engagement to DCN1 in MGC-803 cells and selectively inhibited Cul3/1 neddylation [24]. Compound **5**, which was discovered from in-house structurally diverse molecular library (ca.1000 compounds), inhibited the interaction of DCN1 and UBE2M (IC_{50} = 15 nM) [25]. Compound **5** inhibited the interaction of UBE2M and DCN1 at molecular and cellular levels, resulting in the decrease of cullin3 neddylation and accumulation of its substrates. Compound **6** is a predominant chalcone in the kava plants named Flavokawain A[26]. Compound **6** inhibited NEDD8 conjugations to both Cullin1 and Ubc12 in PC-3 cells and Ubc12 Neddylation *in vitro* assay (**Fig.1**).

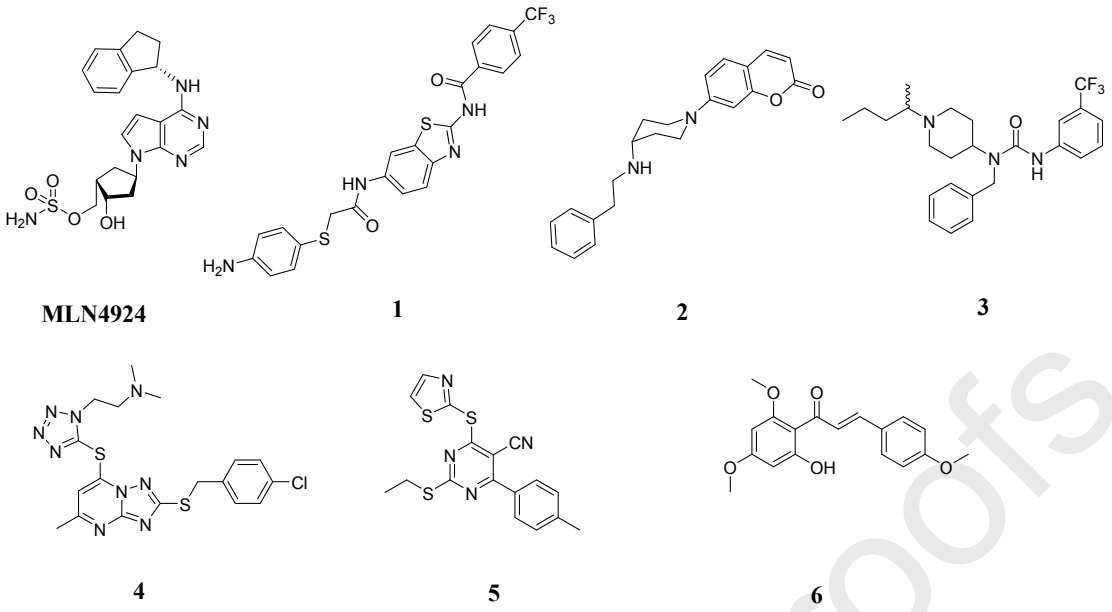


Fig. 2 Structures of reported neddylation inhibitors

In continuation with our efforts toward to the discovery of neddylation inhibitors with potential anticancer activity, we attempt to discover structurally diverse compounds as novel neddylation inhibitors. In this study, by screening our in-house structurally diverse molecular library, we found 1,2,4-triazine scaffold might be a potential scaffold for the inhibition of neddylation by western blotting and molecular docking studies. What's more, we have reported a series of 1,2,4-triazine derivatives as potential anticancer agents[28] and 1,3,5- triazine derivatives were selected out of 100 top-ranked compounds as potential NAE inhibitors by structure-based virtual screening[29]. Thus, the hit 1,2,4-triazine scaffold (compound **IV**) was first selected and its effects on the inhibition of neddylation were identified **(Fig. 3A)**.

Initially, we predicted the binding mode of compound **IV** within the NAE binding site using the reported NAE/ MLN4924 complex (PDB code 3GZN). As shown in **Fig. 3A**, the 1,2,4-triazine core ring of compound **IV** form two hydrogen bonds with the positively charged side chain of Lys124. And compound **IV**, Lys124 and Asp102 make up hydrogen bond networks, which could obviously increase the binding affinity of compound **IV**. The 3,4,5-trimethoxyl-phenyl group is located in a hydrophobic pocket surrounded by Ile75, Ile99, Met101, Ile148, Leu166, Ile170 and Ala171. Meanwhile, the methoxy group form a hydrogen bond with the main chain of Ile148. However, from the docking results, we could find that only a part of the binding pocket is occupied

by compound **IV**. The non-binding sub-pocket provided us a way to optimize our inhibitors based on the compound **IV** and the structure of sub-pocket (**Fig. 3B**). Then, the overall effects of compound **IV** on neddylation were next investigated. The results showed that the formation of Cullin1-NEDD8 was dose-dependently inhibited in MGC-803 cells (**Fig. 3C**). Cullin1 is a substrate protein of neddylation. Therefore, all the findings suggested that compound **IV** might serve as a novel scaffold and might block the neddylation in MGC-803 cells.

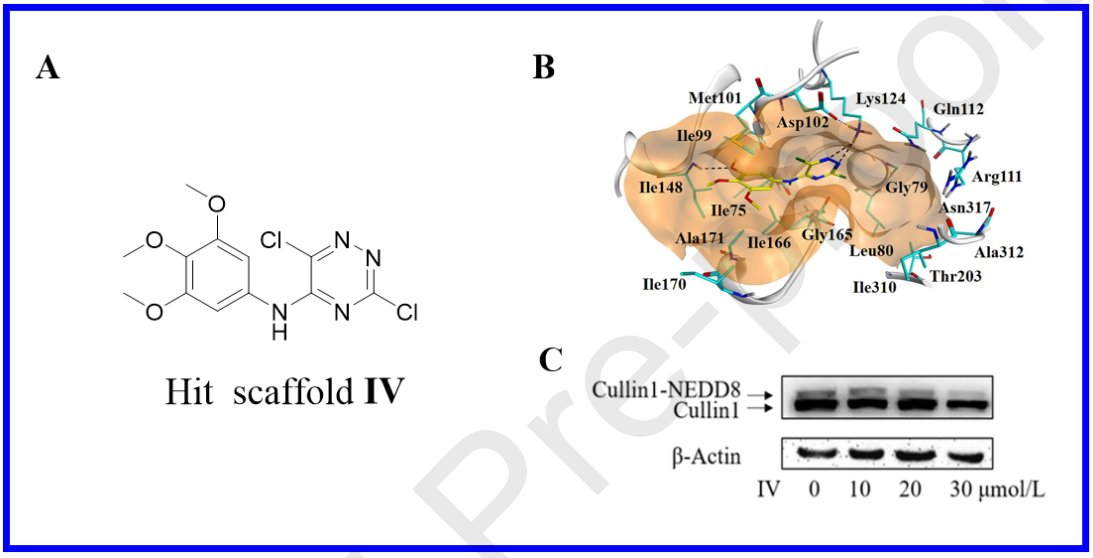


Fig 3. (A) Structure of hit 1,2,4-triazine scaffold (compound **IV**). (B) Docking results of compound **IV** with NAE (PDB code: 3GZN). Compound **IV** is shown in yellow stick model, and key residues of NAE are shown in cyan stick models. The binding pocket is shown in an orange surface. The nitrogen, oxygen, sulfur and chlorine atoms are shown in blue, red, yellow and green, respectively. Hydrogen bonds are shown as black dashed lines. (C) Compound **IV** inhibited neddylation in MGC-803 cells.

Based on above results, a series of novel 1,2,4-triazine derivatives were synthesized based on 1,2,4-triazine scaffold by incorporating bioactive substructures such as aromatic groups and indole groups for better efficacy. What's more, various indoles were designed and synthesized as potential anticancer agents[30-32]. Among them, compound **V11** was investigated the impact of **V11** on neddylation activity (**Fig. 4**).

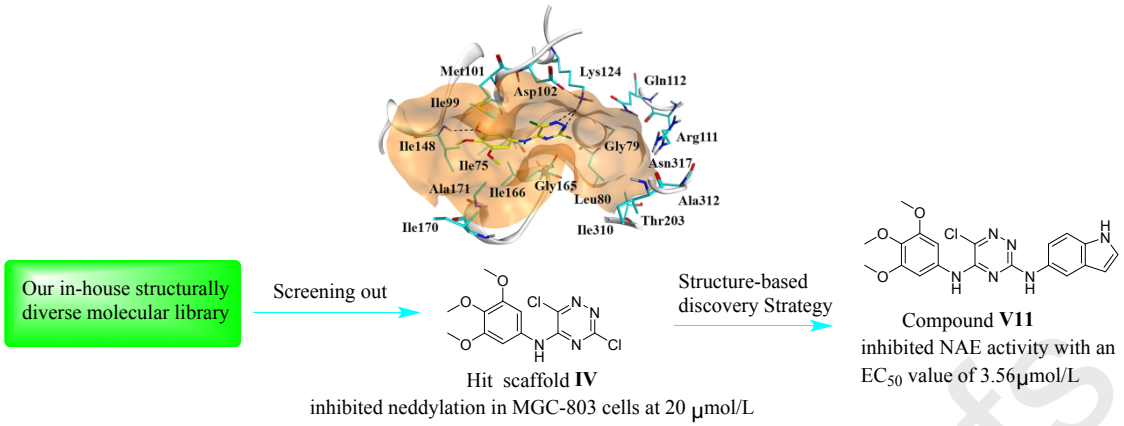
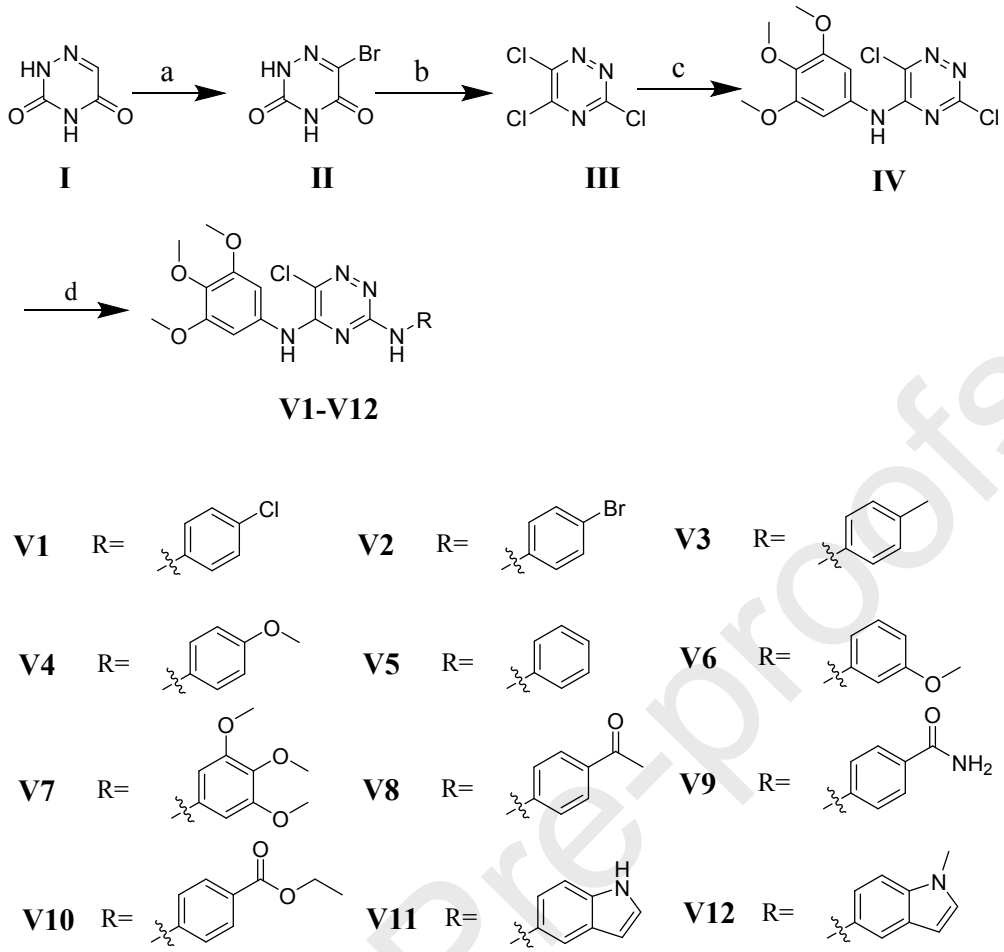


Fig. 4 1,2,4-triazine-based derivatives as novel neddylation inhibitors

The synthetic route of 1,2,4-triazine-based derivatives is shown in **Scheme-1**. Starting from commercially available compound 1,2,4-triazine-3,5(2*H*,4*H*)-dione (**I**), the electrophilic bromine was introduced in the position 6 of the triazinic ring at room temperature to obtain the compound **II**. In the presence of N, N-diethylaniline, phosphorus pentachloride and phosphoryl trichloride, compound **II** was converted to compound **III** at 130°C. The first chlorine atom in the position 5 of intermediate compound **III** was displaced by 3,4,5-trimethoxyaniline in the presence of TEA in THF at 65°C to afford a key compound **IV**. Finally, compound **IV** reacted with substituted arylamines in the presence of camphor sulfonic acid (CSA) in isopropanol to give the target compounds **V1-V12**. Finally, all the target compounds were fully characterized by NMR and HRMS.



Scheme 1. (a) Br_2, H_2O. b) PCl_5, N, N-diethyl aniline, $POCl_3$, 130°C. (c) 3,4,5-trimethoxyaniline, Et_3N, THF, 65°C. (d) arylamines, CSA, isopropanol, reflux.

The antiproliferative activity of all the target compounds was evaluated against three human cancer cell lines (MGC-803, PC-3, and EC-109) using MTT assay method and the well-known anticancer drug 5-fluorouracil as reference drug (**Table 1**).

As shown in **Table 1**, most of compounds exerted moderate to potent cytotoxic activity against three human cancer cell lines. Particularly, compound **V5** and **V11** exhibited the better growth inhibition against the tested cell lines (MGC-803, PC-3 and MCF-7) with IC_{50} values of 8.75 μM, 9.75 μM, 12.57 μM and 8.22 μM, 9.28 μM, 9.64 μM, respectively. The potency of the compounds varies with respect to substitutions on the simple phenyl ring. Comparing **V1-V2** with **V3-V7**, compounds with electron-donating groups at phenyl ring exhibited weaker cytotoxic activity than compounds with electron-withdrawing groups and compound **V5** without substituent group against MGC-803 cells. The relationships between the substituent groups and the

antiproliferative activities were 4-H > 4-Br > 4-Cl > 3-OCH_3>4-OCH_3 > 4-CH_3 > 3,4,5-triOCH_3. Moreover, compound **V11** with the indole group exhibited the best activity against three human cancer cell lines. Importantly, MGC-803 cells were more sensitive to most of the compounds, including compound **V11**, than PC-3 cells and EC-109 cells. Therefore, compound **V11** was chosen to investigate its impact of neddylation activity on MGC-803 cells.

Table 1. The cytotoxicity results of 1,2,4-triazine compounds (**V1-V12**) against human cancer cell lines (MGC-803, PC-3, and EC-109).

Compounds	IC_{50}(μM) [a]		
	MGC-803	PC-3	EC-109
V1	12.59±0.53	9.61±0.22	32.61±2.64
V2	11.35±1.02	8.99±1.32	41.57±1.91
V3	19.79±0.51	14.26±0.51	27.34±2.06
V4	18.95±2.71	13.47±0.62	22.34±1.06
V5	8.75±0.79	9.75±1.46	12.57±1.23
V6	16.46±0.82	11.26±0.31	15.94±5.23
V7	24.31±2.67	34.31±3.88	>50
V8	13.01±0.68	38.63±1.37	36.34±1.76
V9	15.22±1.77	32.15±2.56	42.38±2.13
V10	53.62±3.23	58.45±3.42	46.64±1.25
V11	8.22±0.283	9.28±1.14	9.64±1.47
V12	28.13±1.42	48.48±2.42	42.12±1.84
5-FU	9.79±0.17	20.42±1.83	21.21±3.61

[a]Antiproliferative activity was assayed by exposure for 48 hours.

The impacts of compound **V11** on neddylation activity were investigated. First, a dose–response experiment was performed using neddylation kit *in vitro* which measured the formation of the Ubc12-NEDD8 conjugation products. The inhibition of NAE would decrease the levels of Ubc12-NEDD8 conjugation, therefore the results could be used to evaluate the impacts of compound **V11** on the NAE activity[15]. As shown in **Fig. 5A**, the formation of Ubc12-NEDD8 was almost suppressed in the presence of compound **V11** at 5 μM. MLN4924 also significantly inhibited Ubc12-NEDD8 conjugation at 10 μM (**Fig. 5A**). Therefore, compound **V11** inhibits neddylation (enzyme-based assay).

The ability of compound **V11** to inhibit Ubc12-NEDD8 formation in MGC-803 cells was next evaluated (**Fig. 5B**). MGC-803 cells were incubated with compound **V11** for 24 hours. According to the results, a dose-dependent inhibition of Ubc12–NEDD8 formation was observed upon treatment with compound **V11**. Furthermore, the ability of the compound **V11** to inhibit the levels of Ubc12-NEDD8 conjugation in MGC-803 cells was assessed by Western blotting. The formation of Ubc12-NEDD8 was obviously suppressed at 5μM and nearly complete inhibited at 10μM in the presence of compound **V11**(**Fig. 5B**). The results showed that compound **V11** decreased Ubc12-NEDD8 conjugation in MGC-803 cells in a dose-dependent manner with an EC_{50} value of 3.56 μM (**Fig. 5G**). What's more, the formation of NAE1-NEDD8 and Cullin3-NEDD8 were almost suppressed in the presence of compound **V11** at 10 μM. These results suggested that compound **V11** blocked the neddylation, in which NAE activated NEDD8 and transferred it to Ubc12 and Cullin3.

The neddylation of Cullin1 (another E3 enzyme) was decreased in a time-dependent manner (**Fig. 5D**). The overall effects on neddylation of compound **IV** and **V11** were next investigated. The results indicated that both compound **IV** and compound **V11** inhibited neddylation in MGC-803 cells (the smears of neddylation protein binds were decreased by compounds **IV** and **V11**) (**Fig. 5C**). Furthermore,

compound **V11** had a stronger activity than compound **IV**, which was consistent with the cell viability inhibition results (**Table. 1**). According to these results, compound **V11** inhibited the NAE activity and blocked neddylation in MGC-803 cells, leading to a corresponding decrease in the abundance of E3s. As a result of neddylation inhibition, cell cycle related protein P21 was increased (**Fig. 5E**), and some pro-apoptosis proteins such as ATF4, CHOP, DR5, Noxa were obviously accumulated (**Fig. 5F**) which indicated compound **V11** might have potential anticancer activity.

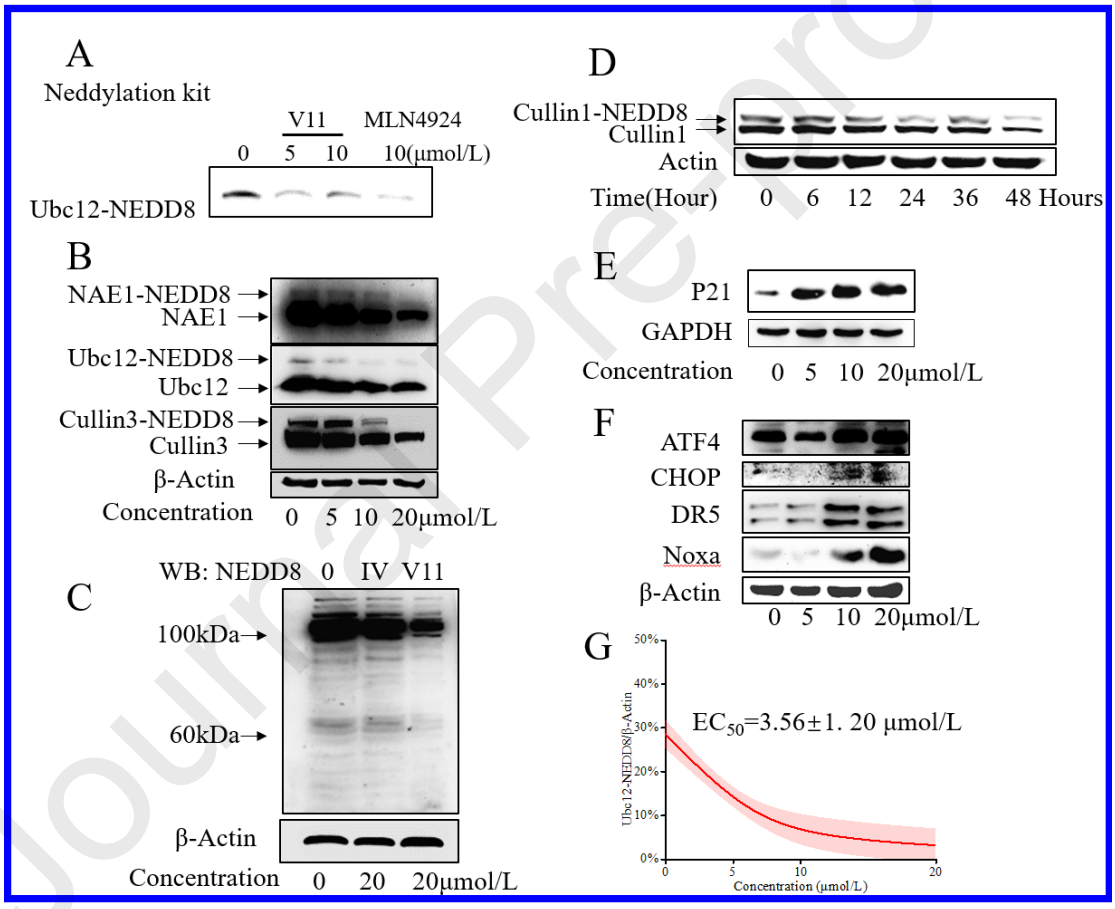


Fig.5 Compound **V11** inhibited neddylation. (A) Compound **V11** inhibited NAE activities in enzyme-based assay. (B, C&D) Compound **V11** inhibited neddylation in MGC-803 cells. (E&F) Level of cell cycle or apoptosis related proteins. MGC-803 cells were treated treated with different concentration of compound **V11** for 24 hours. (G) The quantification of Ubc12-NEDD8 formation was determined by densitometry of triplicate experiments.

The inhibited effects of compound **V11** on proliferation of gastric cancer cell lines

MGC-803 were evaluated. As shown in **Fig. 6A**, the cell viability was decreased in a dose-dependent manner by a 48 hours treatment with compound **V11**, with an IC_{50} value of 8.22 μmol/L. Then, the growth curve of MGC-803 cells was drawn (**Fig. 6B**), and it showed that the compound **V11** inhibited the growth of MGC-803 cells within 48 hours. The treated group increased to 1.3 times while the untreated group grew to approximate 3.7 times. The anti-proliferation activity of compound **V11** was evaluated by colony formation assay. Within a 7 days treatment, the low dose treatment group evidently inhibited the cell colony formation (**Fig. 6C**). These results suggested that the compound **V11** inhibited the proliferation of MGC-803 cells in a dose and time-dependent manner.

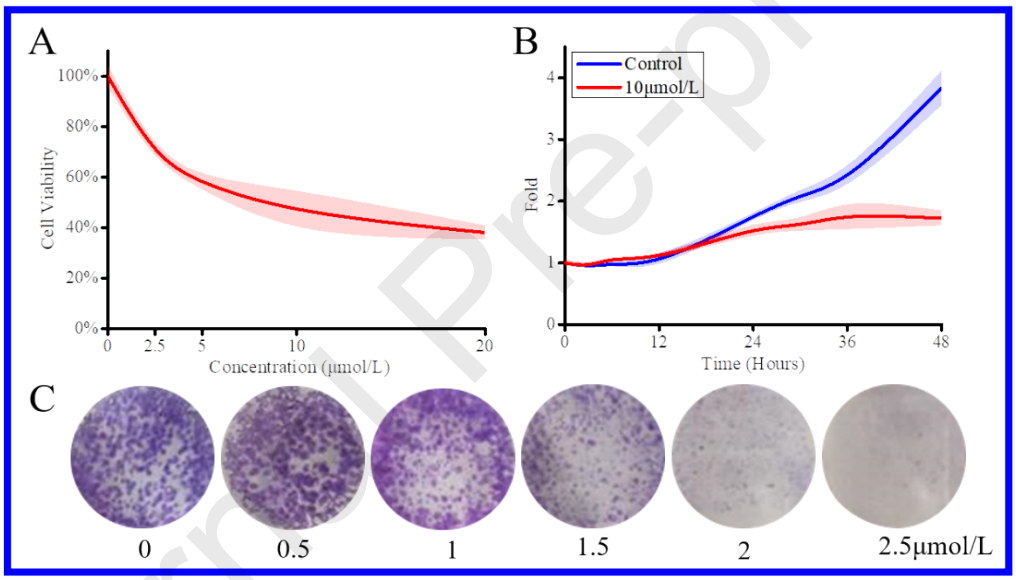


Fig. 6 (A) Cell viability of MGC-803 cells. MGC-803 cells were treated with different concentrations of compound **V11** for 48 hours. (B) Cell growth curve of MGC-803 cells. MGC-803 cells were treated with compound **V11** for different times, MGC-803 cells were treated with DMSO as control. (C) Colony formatting analysis. MGC-803 cells were treated with different concentrations of compound **V11** for 7 days.

To determine the effects of compound **V11** on cell cycle, the flow cytometry assay was performed. As described in **Fig. 7A&B**, the results showed that the MGC-803 cells were arrested in G2/M phase after a 24 hours treatment, the percentage of G2/M cells rose to twice that of the untreated group approximately. Apart from of P21, some other

proteins involved in cell cycle regulation such as CyclinB1 and CDK1 play important roles in G2-M phase transformation. As shown in **Fig. 7C**, CyclinB1 and CDK1 were significantly down-regulated by compound **V11**. Therefore, compound **V11** regulated the cell cycle related proteins P21, CyclinB1 and CDK1, leading to a G2/M phase arrestment.

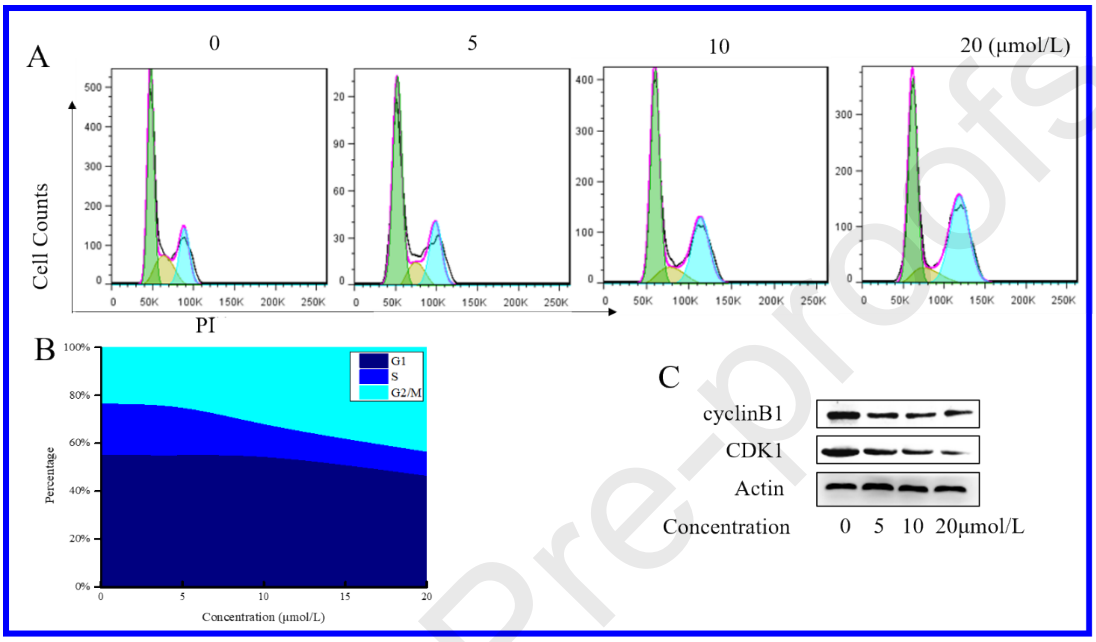


Fig. 7 (A&B) Cell cycle analysis. MGC-803 cells were treated with different concentrations of compound **V11** for 24 hours. (C) Level of cell cycle related proteins. MGC803 cells were treated with different concentrations of compound **V11** for 24 hours.

The accumulations of ATF4, CHOP, DR5 and Noxa may lead to cell apoptosis. Hence, the apoptosis induction of compound **V11** against MGC-803 cells was next investigated. As shown in **Fig. 8 A&B**, the compound **V11** treatment group up-regulated the apoptosis rate to about 60%. The results in **Fig. 8C** showed that the apoptosis related proteins Bax, XIAP, Caspase9, Caspase3 and PARP were evidently regulated. The pro-apoptosis protein Bax was up-regulated, while the anti-apoptosis protein XIAP was down-regulated. Caspase9, Caspase3 and PARP were cleaved by the compound **V11**. All these proteins together with ATF4, CHOP, DR5 and Noxa are involved in extrinsic and intrinsic apoptosis pathways. These results suggested the compound **V11** induced apoptosis via extrinsic and intrinsic apoptosis pathways.

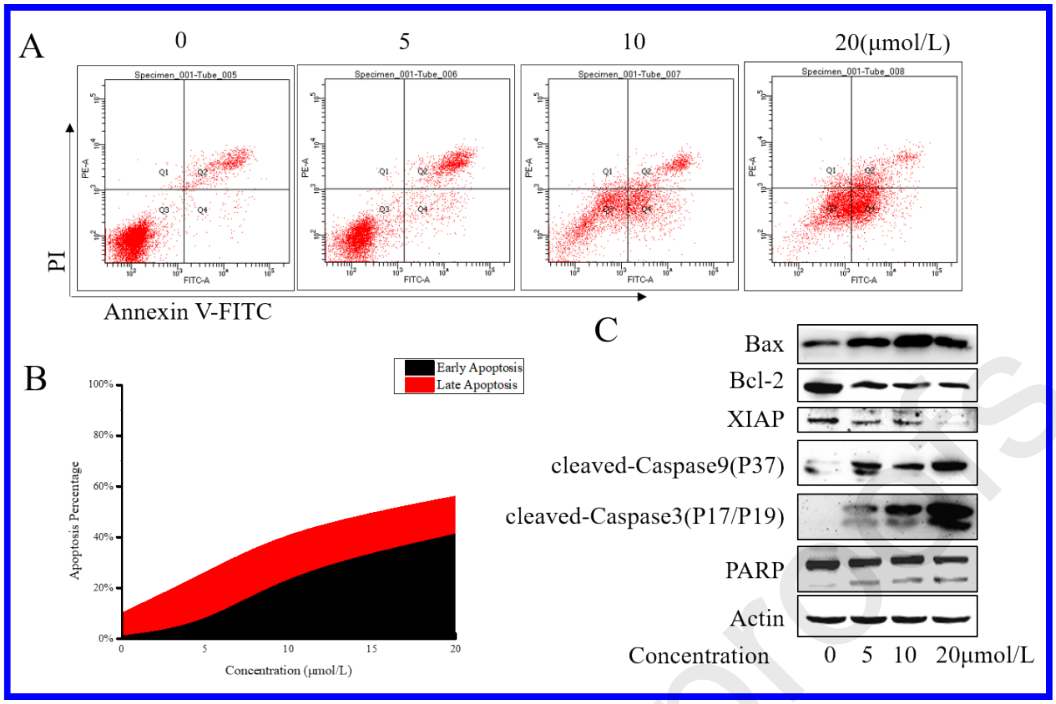


Fig. 8 (A&B) Cell apoptosis analysis. MGC-803 cells were treated with different concentrations of compound **V11** for 48 hours. (C) Level of cell apoptosis related proteins. MGC-803 cells were treated with different concentrations of compound **V11** for 48 hours.

Based on the above experiments, 1,2,4-triazine derivative **V11** showed the inhibition of neddylation and inhibited the NAE activity. Because compound **V11** might be a novel neddylation inhibitor, molecular docking study was carried out using AutoDock Tools 1.5.6 as an automated tool to perform docking and predict the possible binding mode of compound **V11** with NAE (PDB: 3GZN).

The docking study was performed between NAE and most potent compound **V11** (**Fig. 9**). We could find that compound **V11** share a similar binding conformation with compound **IV**, and its 1,2,4-triazine core ring and 3,4,5-trimethoxyl-phenyl group have the same interactions with NAE. Moreover, the nitrogen atom linker could form a hydrogen bond with the main chain of Gly165. The electron rich indole ring could have strong electrostatic interactions with surrounding residues Gly79, Arg111, Gln112 and Asn317. In addition, the indole ring could also have hydrophobic interactions with Leu80, Thr203, Ile310 and Ala312. These additional interactions between NAE and compound **V11** might make compound **V11** bind more tightly to NAE than compound **IV** and also could explain that compound **V11** have a better inhibitory activity of neddylation.

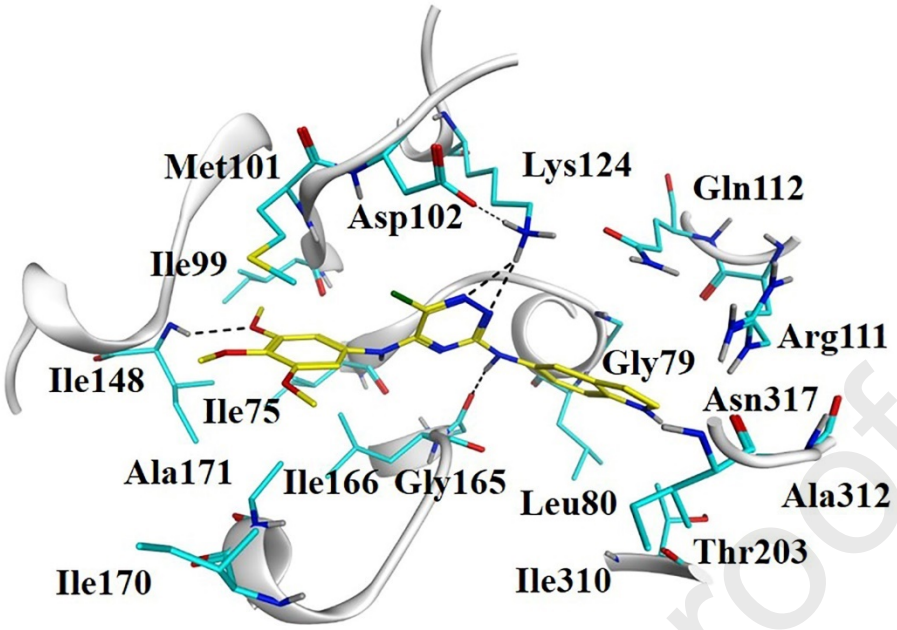


Fig. 9 Docking results of compound **V11** interact with NAE (PDB code: 3GZN). Compound **V11** is shown in yellow stick model, and key residues of NAE are shown in cyan stick models. The nitrogen, oxygen, sulfur and chlorine atoms are shown in blue, red, yellow and green, respectively. The hydrogen bonds are shown as black dash lines.

In conclusion, 1,2,4-triazine scaffold was chosen as a hit scaffold via screening our in-house structurally diverse molecular library further structure-based optimizations. Molecular docking results and the overall effects of compound IV on neddylation suggest that compound **IV** might serve as a novel scaffold and might block the neddylation in MGC-803 cells. Therefore, a series of novel 1,2,4-triazine derivatives were synthesized based on 1,2,4-triazine scaffold by incorporating bioactive substructures such as aromatic groups and indole groups and evaluated for their cytotoxic activity against MGC-803 cells, PC-3 cells and EC-109 cells using MTT assay. The compound **V11** was chosen to investigated its impacts of neddylation activity *in vitro*. Compound **V11** could block neddylation and inhibit the activity of NAE (with an EC_{50} of 3.56 μM), leading to the decrease of UBC12-NEDD8 conjugation. The formation of Cullin3-NEDD8 and Cullin1-neddylation were also inhibited. According to molecular docking results, we could find compound **V11** would bind tightly to NAE via hydrogen bonds and hydrophobic interactions. Compound **V11** possessed the best antiproliferative ability with an IC_{50} value of 8.22μM against MGC-

803 cells. Anticancer activity studies suggested that compound **V11** inhibited MGC-803 cells growth, caused a cell cycle arrestment at G2/M phase and induced apoptosis via extrinsic and intrinsic apoptosis pathways. All the findings suggest that 1,2,4-triazine scaffold might be as a hit scaffold for the development of neddylation inhibitors and compound **V11** might be as a novel NAE inhibitor with potential anticancer activity against MGC-803 cells.

Acknowledgements

This work was supported by the National Natural Sciences Foundations of China (No. 81703541, 81673322) and China Postdoctoral Science Foundation (No. 2018M632812).

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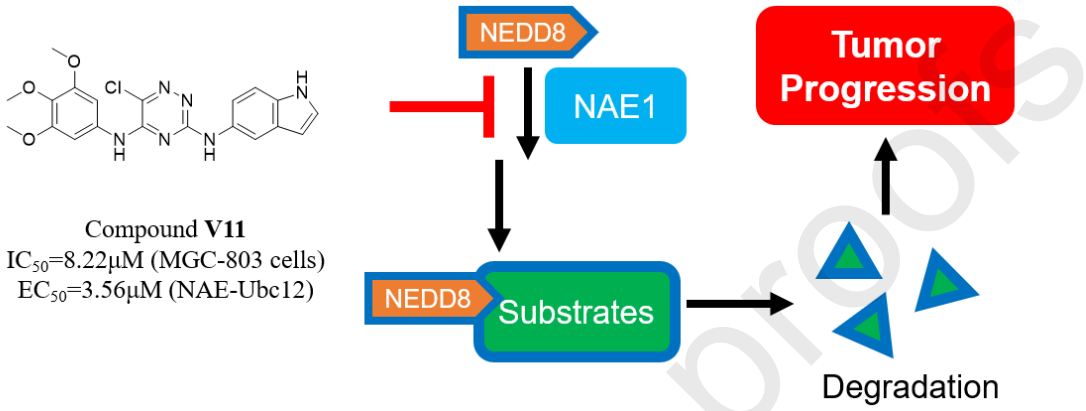
Graphical Abstract



Highlights:

- 1,2,4-triazine scaffold was screened out from in-house structurally diverse molecular library.
- Novel 1,2,4-triazine derivatives as neddylation inhibitors were discovered.
- Compound **V11** could block the neddylation in MGC-803 cells and inhibited NAE activity.
- Among them, compound **V11** exerted the most excellent anti-proliferative activities against MGC-803 cells.
- Compound **V11** caused cell cycle arrest at G2/M phase and induced cell apoptosis.

Declaration of Interest Statement

The authors declare that they have no conflicts of interest.