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A natural product-like inhibitor of NEDD8-activating enzyme
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The natural product-like 6,6′-biapigenin has been identified as only the second inhibitor of NEDD8-activating enzyme using virtual screening. This compound was active in enzyme and cell-based assays, with potency in the micromolar range.

Targeting the ubiquitin-like protein (UBL) NEDD8 pathway has recently emerged as a new strategy for the treatment of cancer. The NEDDylation of the cullin-RING ubiquitin E3 ligases (CRLs) has been shown to be essential for the CRL-mediated ubiquitination of downstream targets in the ubiquitin–proteasome system (UPS). The UPS is vitally involved in protein homeostasis, and inhibitors of the proteosome, such as bortezomib and salinosporamide A, have been demonstrated to be effective anti-cancer agents.

The NEDD8-activating enzyme (NAE) is analogous to the ubiquitin E1 enzyme and is responsible for the activation of NEDD8 and its transfer to Ubc12, the NEDD8-conjugating E2 enzyme. NEDD8 is then conjugated to the cullin proteins of the CRLs, activating their ubiquitin E3 ligase activity. Thus, the specific inhibition of NAE can mediate the rate of ubiquitination and degradation of the subset of proteins regulated by CRLs, including cancer-related substrates such as p-IκBα and c-myc. The recently reported NAE inhibitor MLN4924 has been shown efficacy against both solid and hematological human cancer cell lines (Fig. 1).

The adenine moiety of ATP is located in a predominantly lipophilic region involving hydrophobic residues such as Met101 and Ile148. The adenine ring forms H-bonds with Ile148, Gln149, and Asp167, while the ribose 3′-OH group forms H-bonds with Asp100 and Lys124. In addition, Lys124 is H-bonded to the N-terminal domain of NAE in a similar fashion compared to ATP (see below). MLN4924 is able to form H-bonds that mimic those of ATP. MLN4924 is a nucleotide mimic of adenosine 5′-monophosphate (AMP) and binds to the adenylation domain of NAE in a similar fashion compared to ATP (see below). MLN4924 is able to form H-bonds that mimic those of the NAE–ATP complex. Furthermore, the covalent adduct formed between MLN4924 and C-terminus of NEDD8 has been demonstrated to be the inhibitory species.

To our knowledge, no other NAE inhibitor has yet been reported. High-throughput virtual screening is a powerful and efficient tool for drug discovery and design. We have previously performed high-throughput virtual screening to identify natural product-like and drug-like G-quadruplex ligands, as well as natural product-like inhibitors of the important proinflammatory cytokine tumor necrosis factor-alpha. Meanwhile, natural products provide a valuable array of diverse molecular scaffolds and biologically validated substructures, and they have long been recognized as an important source of new therapeutics. Encouraged by the success of MLN4924, we endeavored to apply our molecular modeling methods to identify natural product or natural product-like small molecule inhibitors of NAE. We used the X-ray crystal structure of the quaternary APPBP1–UBA3–NEDD8–ATP complex for our molecular modeling investigations (PDB: 1R4N).

NAE is a 100 kDa multidomain heterodimer composed of APPBP1 and UBA3 subunits, and its structure resembles a canyon with a large groove in the middle. NEDD8 is positioned in the groove between the heterodimer subunits, with the C-terminus extended towards the ATP binding site. The adenylation domain contains the Gly-X-Gly-X-X-Gly nucleotide binding motif, and ATP binds in this region adjacent to NEDD8. The adenine moiety of ATP is located in a predominantly lipophilic region involving hydrophobic residues such as Met101 and Ile148. The adenine ring forms H-bonds with Ile148, Gln149, and Asp167, while the ribose 3′-OH group forms H-bonds with Asp100 and Lys124. In addition, Lys124 is H-bonded to the z-phosphate group of ATP. MLN4924 is a nucleotide mimic of adenosine 5′-monophosphate (AMP) and binds to the adenylation domain of NAE in a similar fashion compared to ATP (see below). MLN4924 is able to form H-bonds that mimic those of the NAE–ATP complex. Furthermore, the covalent adduct formed between MLN4924 and C-terminus of NEDD8 has been demonstrated to be the inhibitory species. Thus, MLN4924 is considered a mechanism-based inhibitor of NAE.

Over 20 000 compounds from a chemical library of natural product and natural product-like structures were screened in silico. The flexible ligands were docked to a grid representation of the receptor and assigned a score reflecting the quality of the complex according to the ICM method [ICM-Pro 3.6-1d molecular docking software (Molsoft)]. The highest-scoring

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compounds were tested in a preliminary enzyme activity assay and the symmetrical flavonoid dimer 6,6'-biapigenin (I) emerged as the top candidate (Fig. 1).

Dimeric flavonoids are known to display a broad spectrum of biological properties, such as anti-inflammatory, anti-cancer, anti-viral and anti-microbial activities.15 Interestingly, only a few examples of biflavonoids containing the C6–C6' linkage have been isolated from natural sources.16 6,6'-Biapigenin (I) itself has not been found in nature but has been synthesized from 6,6'-biapigenin hexaacetate, in turn obtained from succedanealflavanone (6,6'-binarigenin).16 Derivatives of 6,6'-biapigenin have been reported to inhibit Mycobacterium tuberculosis growth,17 and to inhibit fungal aflatoxin production by the fungus Aspergillus flavus.16 To our knowledge, no biological activity of I has been reported.

Our molecular modeling results show that the binding pose of compound I in the NAE–NEDD8 complex is remarkably different to that of ATP or MLN4924 (Fig. 2). Intriguingly, I is not predicted to occupy the hydrophobic pocket near Met101 and Ile148, nor the ribose binding region located between Asp100 and Asp167 (Fig. 2). Instead, I is situated closer to the APPBP1 subunit in the region normally occupied by the phosphate groups of ATP, and also extends further into the space near NEDD8 (Fig. 2b and c). Consequently, I is unable to form the aforementioned H-bonds that mimic those between ATP and NAE. Instead, I is predicted to make two H-bonds to the side chain carboxylate group of Asp102 through its 7-OH and 7'-OH groups, and a H-bond to the side chain of Asp273 through its 5-OH substituent. The unique binding pose of I allows H-bonding interactions to NEDD8 and APPBP1 not present in the complexes of NAE–NEDD8 with ATP or MLN4924. The 4'-OH group of I is predicted to form a H-bond to the backbone of NEDD8’s C-terminal glycine, while the 4-carbonyl group is able to H-bond with the side chain of APPBP1’s Arg15. The binding score for I with NAE was calculated to be −34.7, compared to −30.3 and −30.8 for ATP and MLN4924 respectively.16 The predicted binding coordinates of ATP in the binding pocket are within 2.0 Å RMSD of the reported values in the X-ray crystal structure.10

Unlike MLN4924, compound I is not a nucleotide mimic, and cannot form a covalent adduct with NEDD8. Thus, I is not expected to be a mechanism-based inhibitor of NAE. Based on the strong calculated binding affinity of I to the active site of NAE, together with the multiple H-bonding interactions with UBA3, APPBP1 and NEDD8, we propose that I may instead act as a reversible ATP-competitive inhibitor of NAE. Our molecular modeling results suggest that compound I can be tentatively considered as a new class of NAE inhibitor. However, further experiments will be required to confirm the exact mechanism of inhibition.

To validate the results of our molecular modeling, a dose–response experiment was performed using an enzymatic assay that measures the NAE-mediated formation of the Ubc12–NEDD8 thioester product. Inhibition of NAE would be expected to lead to decreased levels of the Ubc12–NEDD8 conjugate. A dose-dependent reduction of the intensity of the Ubc12–NEDD8 band was observed upon incubation of NAE with I, with nearly complete inhibition observed at 50 μM (Fig. 3a). Using densitometry analysis, the IC50 value for NAE inhibition by I was estimated to be ca. 20 μM (Fig. 3b).

We next investigated the ability of I to inhibit NAE activity in human cancer cells. Caco-2 (human epithelial colorectal adenocarcinoma) cells were incubated with I for 24 h and analysed for Ubc12–NEDD8 conjugate levels. A dose-dependent inhibition of Ubc12–NEDD8 levels was observed upon treatment with I, with an IC50 = ca. 5 μM (Fig. 4). Nearly complete inhibition was observed at 25 μM of I. This suggests that I is able to inhibit NAE activity in human cells.

Since flavonoids are known as promiscuous binders,19 we sought to determine the selectivity issue by screening I against a panel of 11 kinases.20 At 20 μM of I, 9 out of the 11 kinases showed less than 20% inhibition (Table S1, ESI†).
In conclusion, we have discovered a new inhibitor of NAE from a natural product and natural product-like chemical library using a structure-based design. The identification of 6,6′-biapigenin (1) represents, to the best of our knowledge, only the second example of NAE inhibition by a small molecule. I could inhibit NAE activity in both enzyme and cellular assays, with potencies in the micromolar range. Furthermore, our molecular modeling results suggest a unique binding mode for I that is very different to that of the known NAE inhibitor MLN4924. We tentatively propose that I can be considered as a new class of NAE inhibitors. We are currently elucidating the details of the mechanism of NAE inhibition by I, and are conducting in silico lead optimization to generate further analogues for in vitro biological testing.

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Notes and references

11 Unless otherwise specified, amino acid residues refer to those of UBA3, which is responsible for the majority of non-covalent interactions with small molecules in the NAE binding site.
13 Obtained from AnalytiCon Discovery GmbH. See ESI for details.
18 The binding score for MLN4924 is based on the non-covalent complex of the inhibitor with NAE–NEDD8.
20 Conducted by Caliper Life Sciences Inc. See ESI for details.