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Discovery of small molecule inhibitors for the *C. elegans* caspase CED-3 by high-throughput screening

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Abstract

C. elegans has been widely used as a model organism for programmed cell death and apoptosis. Although the CED-3 caspase is the primary effector of cell death in *C. elegans*, no selective inhibitors have been identified. Utilizing high-throughput screening with recombinant *C. elegans* CED-3 protein, we have discovered and confirmed 21 novel small molecule inhibitors. Six compounds had IC₅₀ values < 10 μM. From these, four distinct chemotypes were identified. The inhibitor scaffolds described here could lead to the development of selective molecular probes to facilitate our understanding of programmed cell death in this model organism.

Keywords

C. elegans; caspase; CED-3; high-throughput screening; small molecule inhibitor; apoptosis

1. Introduction

Apoptosis is a form of programmed cell death necessary for development and cellular homeostasis [1]. The most downstream elements of this highly regulated and conserved process are a class of cysteinyl aspartate proteinases called caspases. [2]. The first caspase family member to be associated with apoptosis was the cell death protein CED-3 of *C. elegans* [3]. Since the cloning of *ced-3* in 1993, a number of caspases have been identified

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from various mammalian and non-mammalian species, including those from human, mouse, zebrafish, *Drosophila melanogaster*, and *C. elegans* [4]. It appears that the number of caspases in *C. elegans* is significantly less than that in mammals. Only four caspase-like genes have been identified in *C. elegans*, including *ced-3*, *csp-1*, *csp-2*, and *csp-3* [5]. Among these four caspases, CED-3 is expressed in most cells throughout *C. elegans* development and is the only caspase that has been shown to be required for apoptosis in *C. elegans*. CED-3 is highly homologous to mammalian caspase-3 [3].

The simplicity of the caspase family in *C. elegans* makes it an ideal model organism to study apoptosis and cell death mechanisms [6]. In *C. elegans*, cell death is modulated by anti-apoptotic CED-9, caspase activator CED-4 and the executioner caspase CED-3 [7], with activation of CED-3 being the final step triggering apoptosis [8]. The onset of programmed cell death in *C. elegans* relies on a delicate interaction between CED-3, CED-4, CED-9, and EGL-1. In addition, CSP-3 has been identified as a negative regulator of CED-3 [9]. CED-3 has also been shown to cleave the Dicer RNase to generate a fragment possessing DNase activity which promotes apoptosis through chromosome fragmentation [10].

Structurally, CED-3 is a CARD-containing caspase that appears to serve both as an initiator and effector caspase. CED-3 is expressed as an inactive form that undergoes autocatalytic processing to remove the pro-domain and generate two active subunits of 10 kDa and 20 kDa, respectively [11, 12]. The resulting large and small subunits form a heterodimer with an active site comprised of residues from both subunits. Two heterodimers then align to form the active tetrameric caspase with two catalytic centers. Like other caspases, CED-3 possesses a catalytic cysteine residue embedded within a highly conserved QACRG pentapeptide motif. This cysteine serves as a nucleophile to first form a covalent thioacyl intermediate between the caspase and substrate, followed by hydrolysis of the amide bond catalyzed by the imidazole ring of a conserved histidine residue [13]. Detailed analyses of CED-3 reveal that its physiological function is to cleave vital substrates such as CED-9 in the cell to induce cell death. As a highly specific protease, CED-3 recognizes a tetrapeptide motif and cleaves its substrates carboxyl to an aspartate residue [12, 14]. Using combinatorial peptide libraries as the substrates, the optimal tetrapeptide substrate for CED-3 was found to be DEXD, similar to that of mammalian caspase-3 and -7 [12].

The conserved cleavage motif (DEVD) for caspase 3 enzymes has allowed the development of fluorogenic based assays [15, 16] to identify inhibitors. For example, human caspase-3 (HCASP3) has been associated with neurodegenerative disease [17] and inhibitors to this target have been identified (reviewed in [18]). Peptides and peptidomimetic compounds based on the recognition motif are potent caspase inhibitors but their use has been limited because of their poor bioavailability [19]. RNA aptamers targeting CED-9 have been shown to promote cell killing via a CED-3-dependent mechanism [20]. Carcinogenic benzenoid compounds have been shown to act as inhibitors of apoptosis in *C. elegans* [21], with some reports of direct binding to CED-3 [22]. Selective small molecule inhibitors of CED-3 that may serve as molecular probes [23, 24] to further elucidate the apoptotic cascade in *C. elegans* have not been reported.

In this study, we describe the high-throughput screening of CED-3 with a 28,800-compound library of small molecules and the identification of several potent inhibitors with a subset having selectivity over the closely related HCASP3.

2. Materials and methods

2.1 Reagents

Human Caspase 3 (HCASP3) (catalog # 1083-5) was purchased from Biovision Inc. (Mountain View, CA). Fluorogenic substrate Ac-DEVD-AMC and caspase-3 inhibitor Ac-DEVD-CHO were purchased from BD Pharmingen (San Diego, CA). Costar 384-well, flat-bottom, black plates were from Corning Incorporated (Corning, NY).

2.2 Recombinant *C. elegans* CED-3 expression and purification

C. elegans CED3 full-length cDNA was cloned into the pET-3a FLAG vector and transformed into *E.coli* B21 cells. Cells were grown at 37 °C in LB media and when in log phase CED3 expression was induced by the addition of 0.5 mM IPTG for 3 h. Cells were harvested, lysed and the resulting supernatant cleared by centrifugation to obtain lysate (termed CED-3L). FLAG-CED-3 was purified from bacterial cell lysate by capture on anti-FLAG antibody agarose resin (Sigma) followed by elution using free FLAG peptide (Sigma). The purity of soluble recombinant FLAG-CED-3 was confirmed by SDS-PAGE (data not shown). The proteolytic activities of CED-3L and purified CED-3 were characterized by using the CED-3 assay.

2.3 CED-3 assay

The enzymatic activity of CED-3 was measured by following cleavage of the fluorogenic substrate Ac-DEVD-AMC at room temperature. The assay buffer contained CED-3 (1 μ L CED-3L lysate or 0.5 nM purified CED-3), 50 mM HEPES pH 7.2, 50 mM NaCl, 0.1% CHAPS, 10 mM EDTA, 5% glycerol, 10 mM DTT. Reactions were initiated by adding 20 μ M Ac-DEVD-AMC substrate and reading the increase in fluorescence over time using the Ex₃₆₀/Em₄₆₀ filter set on a Pherastar Plus microplate reader (BMG LABTECH Inc., Cary, NC). The same conditions were used for the assay for HCASP3.

2.4 Chemical libraries and compound handling

A small molecule library of 28,800 compounds was purchased from Asinex Corp (Moscow, Russia). All compounds adhere to Lipinski's rule of 5 [25], were registered in ActivityBase (IDBS Inc., Guildford UK) and could be identified in bar-coded 384-well plates with associated SD file data. Compounds were stored at -20°C in 100% DMSO.

2.5 High-throughput screening of CED-3

A high-throughput screen for small molecule inhibitors of CED-3 activity was adapted from the fluorogenic CED-3 assay described above. All steps were carried out at room temperature. Compounds (1 mM in DMSO) were spotted (0.5 μ L) into columns 3–22 of dry Costar black flat bottom 384-well assay plates using a Biomek® NX (Beckman Coulter Inc., Fullerton, CA). Columns 1, 2, 23, and 24 were spotted with 0.5 μ L of DMSO for use as

controls. For primary screening, CED-3L (25 μ L) was added to the plates using a 384-well Multidrop liquid dispenser (Thermo Scientific, Waltham, MA) and after a 15 min incubation with compounds, the reaction was initiated by the addition of Ac-DEVD-AMC substrate (25 μ L) to yield the following final concentrations: 20 μ M Ac-DEVD-AMC and 10 μ M compound for single-point screening. Minimum (columns 1 and 2; + Ac-DEVD-CHO at 10-fold its IC₅₀ value) and maximum (columns 23 and 24; DMSO only) signal controls were included on every plate. After 60 min, reactions were halted with SDS (0.05% final) and fluorescence measured as above. Inhibition data was analyzed in ActivityBase using Excel.

For dose-response measurements, 10 point 2-fold serial dilutions of the compounds of interest (10 mM stock concentrations in DMSO) were carried out in DMSO using the Biomek NX. Compounds (0.5 μ L) were spotted from the dose response plates into 384-well plates and the assay carried out as above. IC₅₀ values were determined as a function of relative fluorescence units versus inhibitor concentration. Compounds were tested in at least two independent experiments. For the CED-3 assay, the maximum concentration of DMSO is 1% and this concentration of DMSO did not affect the activity of CED-3 (data not shown).

2.6 Estimation of Assay Quality

As an estimate of assay quality, a Z' factor [26, 27] was determined from the means and standard deviations of whole 384-well plates of maximum (Max) signal (enzyme and substrate) and minimum (Min) signal (enzyme, substrate and inhibitor). Z' factor was calculated using the formula: $Z' = 1 - (3SD_{Max} + 3SD_{Min}) / (Mean_{Max} - Mean_{Min})$, where 3SD = three standard deviations. Excel and Prism 5 (GraphPad, San Diego, CA) were used to perform the calculations and statistical analyses needed to determine the Z' factor. Acceptable criteria for HTS were Z' scores > 0.5 and coefficient of variation (CV) < 10% [27].

2.7 Statistical analysis

Prism 5 software was used for nonlinear regression analysis. IC₅₀ values and Hill slopes were determined using a four-parameter dose-response (variable slope) fit.

3. Results

3.1 CED-3 HTS assay optimization and validation

We expressed recombinant *C. elegans* CED-3 in *E. coli* as a FLAG-tagged fusion protein. However, yields of purified FLAG-CED-3 from the lysate using anti-FLAG affinity capture were low and insufficient for HTS. Therefore, we assessed whether the crude lysate (termed CED-3L) would provide a suitable source of active CED-3 for HTS by testing cleavage of a specific caspase-3 substrate. CED-3L activity was measured using a fluorogenic assay monitoring the cleavage of Ac-DEVD-AMC in the presence and absence of the potent and selective caspase-3 inhibitor Ac-DEVD-CHO. Ac-DEVD-CHO inhibited CED-3L with an IC₅₀ value of 1.90 nM and a calculated Hill slope = -0.994 (Fig. 1A). Sufficient purified CED-3 was obtained to allow assay validation and for confirmation studies of HTS actives. A comparable IC₅₀ value of 2.7 nM was obtained for inhibition of purified CED-3 by Ac-DEVD-CHO (Fig. 1B) indicating that the crude lysate fraction could be used as the source

for CED-3 enzymatic activity. Further, a comparable IC_{50} value of 1.98 nM was determined for Ac-DEVD-CHO inhibition of commercially available human caspase-3 (HCASP3) (Fig. 1B).

The CED-3 inhibition assay was adapted to 384-well format with a final assay volume of 50 μ L. To facilitate HTS, the assay was simplified into two steps and CED-3L activity measured as an end point assay after 60 mins by halting the reaction with SDS (the final automation protocol is detailed in Supplementary Table 1). For HTS validation, the CED-3 assay was run in full automation with whole 384-well plates of maximum (CED-3 + substrate with DMSO only) and minimum (CED-3 + substrate with Ac-DEVD-CHO inhibitor at 10-fold its IC_{50} value) signal controls (Supplementary Fig. 1). To assess assay variability, the Z'-factor computation incorporates both the dynamic range of the assay and well-to-well variability and measures both assay quality and performance [26]. The Z' for individual plates was 0.60, with CVs of <10% (Supplementary Fig. 1) demonstrating a suitable assay window and acceptable variability for HTS [27].

3.2 CED-3L HTS for inhibitor identification

To identify inhibitors of CED-3 an Asinex compound library of 28,880 small molecules was screened at 10 μ M single point concentration. CED-3 inhibition values (%) were plotted for each compound (Fig. 2A). Seventy-five (75) active compounds exhibiting CED-3 inhibition at or above a 50% threshold were identified, corresponding to a primary hit rate of 0.26%. Compounds identified as single-point actives were first confirmed using the assay with CED-3 lysate (CED-3L) for dose response/ IC_{50} determination. Dose-response data was loaded in ActivityBase and plots assessed for IC_{50} and Hill slope values. IC_{50} values were determined for the 34 compounds that achieved 50% inhibition of both crude CED-3L and purified CED-3 (data not shown). Of the 34 active compounds, 21 exhibited sigmoidal inhibitor dose-response curves with purified CED-3 (summarized in Fig. 2B). Among the 21 confirmed compounds (28% of the original 75 hits), IC_{50} values on purified CED-3 ranged from 0.75 to 31 μ M. Six compounds had IC_{50} values < 10 μ M and the 10 most potent inhibitors are listed in Table 1 with their Asinex IDs, IC_{50} values on CED-3L and purified CED-3, and Hill slope values. Fig. 3A shows the dose-response inhibition curves for the three most potent inhibitors of purified CED-3, including BAS 01202237 (IC_{50} = 0.75 μ M); BAS 00519146 (IC_{50} = 1.5 μ M) and BAS 05223612 (IC_{50} = 3.9 μ M).

3.3 CED-3 chemotype identification and activity versus HCASP3

We evaluated structural features of the 21 confirmed compounds by performing K-means clustering in order to group compounds with at least 60% similarity. Eight of the 21 compounds were grouped into one of four chemotype classes based on their structural similarity (Fig. 3B). The remaining thirteen confirmed inhibitors were structurally unrelated and had IC_{50} values ranging from 3.9 to 43.4 μ M. For all 21 confirmed CED-3 inhibitors, Supplementary Table 2 summarizes Asinex IDs, molecular structures, IC_{50} values for purified CED-3 and HCASP3, Hill slope values and chemotype.

To assess selectivity of the *C.elegans* CED-3 hits versus the human caspase-3 (HCASP3), the CED-3 hits were tested for dose-response in a comparable assay using commercially

available purified HCASP3. The assay for human CASP3 was first validated using procedures previously described for the CED-3 assay with Ac-DEVD-CHO inhibiting purified HCASP3 with an IC_{50} value of 1.98 nM (see Fig. 1B). The corresponding IC_{50} values versus the HCASP3 are shown in Supplementary Table 2. Of the 21 confirmed CED-3 hits, seven compounds were active on HCASP3 with the remainder showing no inhibition of HCASP3 at the concentrations tested. Interestingly, BAS 00519146, the second most potent cluster 4 inhibitor, is more potent against CED-3 than HCASP3 (IC_{50} CED-3 = 1.5 μ M versus IC_{50} HCASP-3 = 10.8 μ M) (Fig. 3C). Differences in binding affinity to HCASP-3 versus CED-3 are likely due to the fact these two enzymes share only 50% sequence identity.

4. Discussion

The nematode *C.elegans* has long been utilized as a model organism to understand programmed cell death and apoptosis. Activation of the caspase CED-3 is the final step in the apoptotic cascade in *C.elegans* and the availability of selective small molecule inhibitors to the apoptotic machinery in *C.elegans* would be invaluable as molecular probes to dissect apoptotic signaling pathways. The current study describes how novel small molecule inhibitors targeting the *C.elegans* caspase CED-3 were identified from high-throughput screening. First, as it proved challenging to generate sufficient purified CED-3 for HTS, we demonstrated that crude lysate from *E.coli* expressing the recombinant CED-3 could be used as the source of active enzyme for the screen. Typically purified enzyme would be the optimal choice for any assay [28]. The availability of both a highly selective substrate that we hypothesized only CED-3 but not bacterial proteases can cleave, and a selective inhibitor, allowed us to validate the assay using the crude lysate as a source for active CED-3. IC_{50} values obtained from the crude lysate matched closely with those using the limited amount of the purified CED-3 we produced. Further, a Hill slope of very close to 1.0 was obtained with the crude lysate preparation of CED-3 demonstrating *enzymatic* purity. A Hill slope vastly different from 1.0 would be indicative of the presence of other enzyme activities [27]. These findings demonstrate the utility of the fluorogenic assay format for HTS [15, 29–32].

Developing inhibitors to caspases using peptides and peptidomimetic compounds has been challenging because of their poor bioavailability [19]. Therefore, we screened a library of small molecules with drug-like properties [25] for their effectiveness in inhibiting the enzymatic cleavage of the fluorogenic caspase-3 substrate Ac-DEVD-AMC as a means to identify potential CED-3 inhibitors. Out of the compound library of 28,800 small molecules, we initially identified 75 actives; 21 of which were confirmed by dose-response with the purified CED-3 enzyme. The relatively low confirmation rate may indicate a number of false positives interfering with this fluorogenic assay or the challenges with using crude lysate for HTS. Potentially for the latter, if additional HTS is undertaken, this could be addressed by using either a “negative lysate control” (i.e. lysate with no CED-3 expression) or testing the compounds in the presence of lysate expressing a catalytically inactive CED-3 mutant. In general, the hit compounds were more potent on the purified CED-3 versus the crude lysate preparation suggesting potential compound stability or solubility issues with using crude lysate. The identification of four distinct chemotypes shows the ability of the assay to mine structurally related compounds from a diverse set of molecules.

Benzenoid chemicals, in particular benzoquinones, have been shown to directly inhibit caspase-3 by oxidation of the catalytic cysteine [21]. From the 21 confirmed hits, we identified several that may potentially lead to a benzoquinone-like state via common metabolic transformation *in vivo* (e.g. by demethylation or hydroxylation), including members of clusters 1, 2 and 4 that have *p*-substituted aromatics. In particular, of the hits, BAS 05223612 already includes a benzoquinone structural motif. In contrast, because some hits that have the *p*-substituted aromatic substructure also have a fluorine substitution at this position, they are not reasonably anticipated to form a benzoquinone-like metabolite (e.g. BAS 02559443 of cluster 2) and these might present an alternate mechanism of inhibition. Further, structurally, chemotype 3 is distinct from the benzoquinone motif and would potentially inhibit CED-3 by a unique mechanism.

CED-3 shares sequence homology and function in terms of active site inhibitor binding residues with the HCASP3 [33]. Hence, we also assessed the confirmed CED-3 hits for inhibition of HCASP3. We identified CED-3 inhibitors with >10-fold selectivity over HCASP3 and some that were equally potent or more so on the human enzyme. For example, inhibitors from chemotype 4 inhibited both CED-3 and HCASP3 whereas those from chemotypes 1 and 2 only inhibited CED-3. As might be expected the compound possessing the benzoquinone motif (BAS 05223612) inhibited both CED-3 and HCASP3. Two compounds, BAS01847186 and BAS0261429, were as potent or more so on HCASP3 compared to CED-3. These data suggest that the CED-3 inhibitors we identified have potentially differing modes of binding.

Small molecule probes to CED-3 would have utility in dissecting the various mechanistic roles of CED-3. Interestingly, CED-3 has been shown to also have non-apoptotic functions in *C.elegans* [34], including a role in axon regeneration [35]. Further, targeting caspases in pathogenic nematodes with selective small molecules may have therapeutic utility [36]. In summary, using a validated high-throughput screen we have identified novel small molecule inhibitors of CED-3, that in the future, have the potential to be developed as molecular probes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- The validation of crude lysate as a source for active recombinant CED-3 in HTS.
- 21 confirmed hits and 4 distinct chemotypes inhibiting CED-3 were identified.
- A subset of CED-3 hits had selectivity over human caspase-3.
- Several of the CED-3 hits differ structurally from the benzoquinone motif.
- These CED-3 hits have the potential to be developed as selective molecular probes.

Fig. 1. Dose response analysis of the caspase inhibitor DEVD-CHO on *C.elegans* CED-3 and human caspase-3 using a fluorogenic assay

Caspase activity was determined in a fluorogenic assay using the substrate Ac-DEVD-AMC.

(A) The gene for *C. elegans*, CED-3, was expressed in *E. coli* and the crude lysate used as the source for CED-3 enzymatic activity (CED-3L). Data points represent the average CED-3 activity (RFU) per concentration of the inhibitor Ac-DEVD-CHO and error bars show SD (n = 16). (B) Normalized dose response inhibition curves for purified CED-3 and human caspase-3 (HCASP3) by Ac-DEVD-CHO.

Fig. 2. High-throughput CED-3 inhibitor screening

(A) Compounds from the Asinex set were screened at single point 10 μ M concentration versus CED3-L. The scatterplot shows percent inhibition for all compounds screened. Compounds showing >50% inhibition were selected for confirmation. (B) Summary of CED-3 inhibitor screening.

Fig. 3. Dose response curves for select hits and chemotypes

(A) Dose response curves and IC_{50} values for the top three hit compounds on purified CED-3. (B) Chemical structures for the four chemotypes and associated CED-3 versus HCASP3 IC_{50} values for chemotype 4. (C) Dose response curves and IC_{50} values for representative compound from chemotype 4 on purified CED-3 and HCASP3. Values represent the mean of at least two independent assays.

Table 1

Inhibitory activity of the 10 most potent confirmed inhibitors from chemical library screen of *C. elegans* CED-3

Asinex Identifier ^a	CED-3L IC ₅₀ (μM) ^b	Purified CED-3 IC ₅₀ (μM) ^c	Purified CED-3 Hill Slope Value ^d
BAS 01202237	11.1	0.75	1.5
BAS 00519146	19.6	1.5	1.6
BAS 05223612	24.5	3.9	1.7
BAS 00835000	30	4.9	1.38
BAS 02712220	13.5	7.7	0.9
BAS 00253468	21	9	1.79
BAS 02859639	27.1	10.9	0.69
BAS 01988614	43.1	13.6	0.62
BAS 02712108	41.5	13.6	1.21
BAS 00187854	34.7	14.6	1.27

^a Chemical identifier number provided by Asinex Corporation. Compounds are listed by potency on purified CED-3.

For IC₅₀ determinations, serial dilutions of compounds were tested starting at a high concentration of 80 μM^b or 20 μM^c.

^d Hill slope values determined by XLfit in Activity base.