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Letter to the Editor

z-VAD-fmk inhibits peptide:*N*-glycanase and may result in ER stress

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Dear Editor,

After insertion into the endoplasmic reticulum (ER), many proteins are glycosylated and participate in chaperone-assisted folding by the lectins calnexin and calreticulin.[1] A failure to degrade misfolded proteins, such as occurs in protein folding diseases or caused by small molecules that interfere with normal protein maturation in the ER (e.g. DTT, thapsigargin, tunicamycin), results in ER stress. Connections between ER stress and caspase activation have been observed in mouse and human cells for both caspase-4[2,3] and caspase-12.[4,5] The list has recently been expanded to include the initiator caspase-2, and effector caspases-3, and -7, which are activated by depletion of Ca^{2+} stores in the ER.[6] The steps involved in the overall degradation pathway(s) include dislocation of the misfolded glycoprotein from the ER to the cytosol, followed by deglycosylation, ubiquitination, and proteasomal proteolysis. Deglycosylation occurs in the cytosol and is carried out by peptide:*N*-glycanase (PNGase). This enzyme cleaves the β-aspartyl-glucosamine bond between the first *N*-acetyl-glucosamine (GlcNAc) of the glycan and the amide side chain of asparagine, converting the asparagine to an aspartate residue. PNGase has been shown to discriminate between folded and unfolded glycoproteins and to deglycosylate only unfolded glycoproteins.[7]

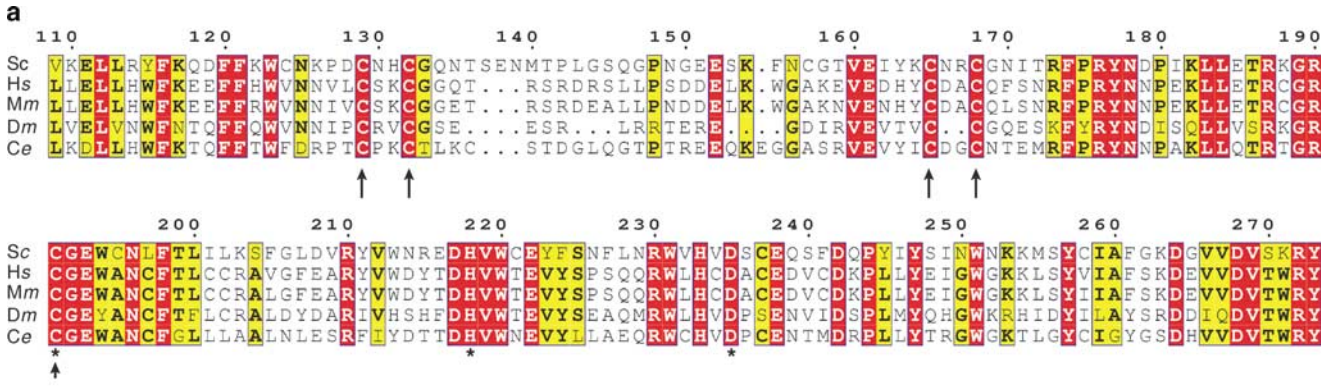
PNGases have conserved residues essential for function.[8] These include a putative cysteine–histidine–aspartate catalytic triad and four additional cysteines, each in a C-X-X-C motif (Figure 1a). We recently identified the general caspase inhibitor, z-VAD-fmk (Figure 1b), as a potent inhibitor of yeast and mammalian PNGase.[9] Complete inactivation of PNGase is afforded at concentrations lower than those typically used for caspase blockade. We found that more selective tetrapeptide fmk inhibitors also blocked PNGase *in vitro*. To better understand the crossreactivity of z-VAD-fmk and related inhibitors with caspases and PNGase we sought to determine the site(s) of modification of PNGase by z-VAD-fmk.

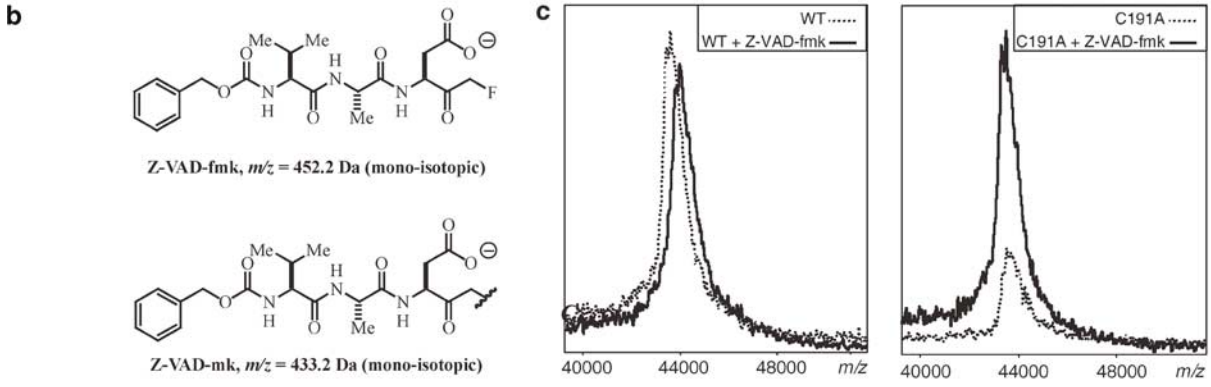
Fluoromethylketones react with cysteine proteases like caspases to yield a covalent thioether adduct.[10] Yeast PNGase (YPng1) contains 14 cysteines, five of which are absolutely conserved. Mutation of any of the putative active site residues – Cys191, His218 and Asp235 – or the other four conserved cysteines – Cys129, Cys132, Cys165 and Cys168 – results in loss of enzymatic activity.[8] Although mutation of the proposed active site cysteine to alanine (C191A) indeed renders PNGase inactive,[8,9] it is not clear whether inactivity is a consequence of eliminating the active site nucleophile or distortion of the active enzyme's conformation. Conformational distortion is suggested by an altered circular dichroism spectrum for the mutant.[8]

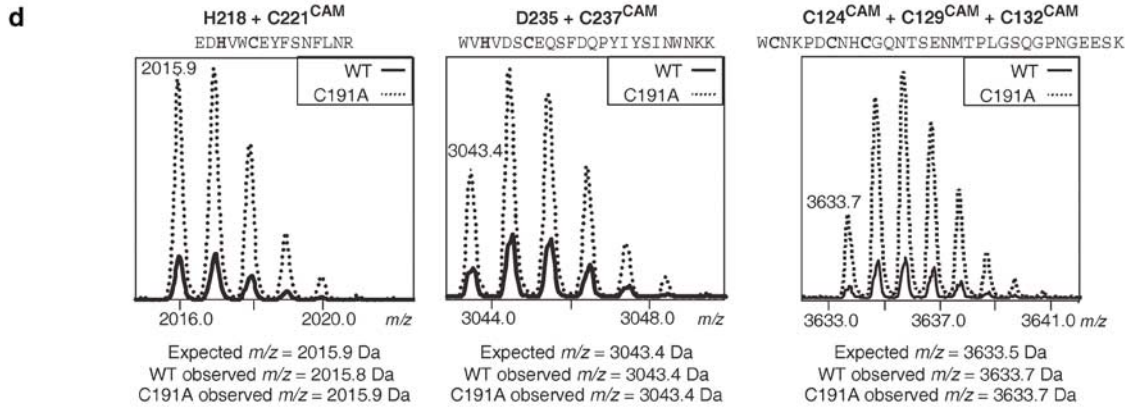
To determine the site(s) of adduct formation between z-VAD-fmk and YPng1, we used mass spectrometry. Following incubation of z-VAD-fmk (Sigma) with either wild-type YPng1 (WT) or the C191A mutant, the proteins were denatured, reduced and alkylated with iodoacetamide to afford carboxamidomethyl (CAM) modification of cysteine thiols. MALDI-TOF analysis shows a mass increase of 392 daltons (Da) (Figure 1c, left panel), for WT, consistent with the addition of a single z-VAD-fmk molecule per YPng1. The difference between the calculated mass increase for a 1 : 1 adduct (433 Da) and the observed increase (392 Da, 0.09% error) is the result of measurement on the full-length protein ($\sim$44 000 Da). Small differences ($<$0.1% error) are common at higher mass ranges. Under identical conditions, no mass increase was observed for the C191A mutant.

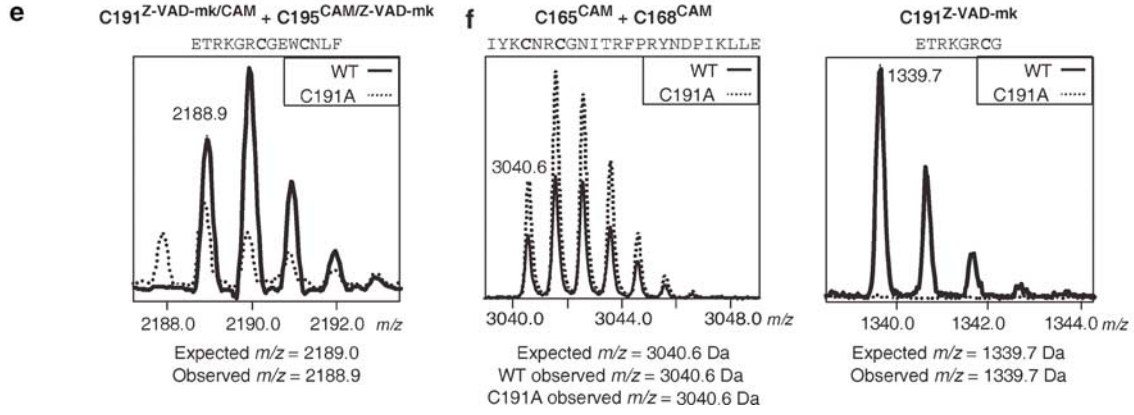
To map the site(s) of modification, z-VAD-fmk-treated WT and C191A were digested with trypsin, chymotrypsin or endo Glu-C peptidases and analyzed by MALDI-MS. Tryptic digests revealed peptides of *m/z* 3043.4 and 2015.9 Da, corresponding to unmodified peptides containing Asp235 and His218, respectively (Figure 1d, left and center panels). We observe no evidence for z-VAD-fmk modification of these peptides. This result shows that putative catalytic triad residues, His218, and Asp235, are not modified by z-VAD-fmk. Additionally, we observed peptides mapping conserved cysteine residues Cys129 and Cys132, each of which was found only as a CAM adduct (Figure 1d, right panel). We also observed seven additional nonconserved cysteine residues, each of which was modified only with CAM. We did not observe any tryptic peptides containing Cys63, Cys195, or the conserved cysteine residues Cys165, Cys168, and Cys191.

Digestion with chymotrypsin yielded peptides mapping 12 of 14 cysteines, with only Cys165 and Cys168 remaining unaccounted. Comparison of WT and C191A chymotryptic digests revealed the detection of a peptide, of *m/z* 2188.9 Da, unique to the WT digest (Figure 1e). This peptide contains Cys191 and Cys195, and the observed mass indicates modification by one each of z-VAD-mk and CAM. Peptides corresponding to these same peptide sequences but lacking z-VAD-mk modification were not observed. As we observe CAM modification of the other 10 cysteine residues that were mapped in the chymotryptic digest, and find no evidence for their modification by z-VAD-fmk, we concluded that the site(s) of WT modification by z-VAD-mk occurs at one or more of the


a
110 120 130 140 150 160 170 180 190
Sc VKELLRYFKQDFFKWCNKPDCNHCGQNTSENMTPLGSQGPNGEESK.FNCGTVEIYKCNRCGNITRFPRYNDPIKLLETRKGR
Hs LLELLLHWFKEEFFHWVNNVLCSKCGGQT...RSRDRSLLPSDDELK.WGAKEVEDHYCDACQFSNRFPRYNNPEKLLETRCGR
Mm LLELLLHWFKEEFFRWVNNIVCSKCGGET...RSRDEALLPNDDELK.WGAKNVENHYCDACQLSNRFPRYNNPEKLLETRCGR
Dm LVELVNWFNTQFFQWVNNIPCRVCGSE....ESR...LRRTERE.....GDIRVEVTVC..CGQESKFYRYNDISQLLVSRKGR
Ce LKDLLHWFKTQFFTWFDRPTCPKCTLKC...STDGLQGTPTREEQKEGGASRVEVYICDGCNTEMRFPRYNNPAKLLQTRTGR
200 210 220 230 240 250 260 270
Sc CGEWCNLFTLILKSFGLDVRYVWNREDHVWCEYFSNFLNRWVHVDSCEQSFDQPYIYSINWNKKMSYCIAFGKDGVVDVSKRY
Hs CGEWANCFTLCCRAVGFEARYVWDYTDHVWTEVYSPSQQRWLHCDACEDVCDKPLLYEIGWGKKLSYVIAFSKDEVVDVTWRY
Mm CGEWANCFTLCCRALGFEARYVWDYTDHVWTEVYSPSQQRWLHCDACEDVCDKPLLYEIGWGKKLSYIIAFSKDEVVDVTWRY
Dm CGEYANCFTFLCRALDYDARIVHSHFDHVWTEVYSEAQMRWLHVDPSENVIDSPLMYQHGWKRHIDYILAYSRDDIQDVTWRY
Ce CGEWANCFGLLLAALNLESRFIYDTTDHVWNEVYLLAEQRWCHVDPCENTMDRPLLYTRGWGKTLGYCIGYGSDHVVDVTWRY


b
Z-VAD-fmk, m/z = 452.2 Da (mono-isotopic)
Z-VAD-mk, m/z = 433.2 Da (mono-isotopic)
c
WT
WT + Z-VAD-fmk
C191A
C191A + Z-VAD-fmk
40000 44000 48000 m/z


d
H218 + C221CAM
EDHVWCEYFSNFLNR
2015.9
WT
C191A
2016.0 2020.0 m/z
Expected m/z = 2015.9 Da
WT observed m/z = 2015.8 Da
C191A observed m/z = 2015.9 Da
D235 + C237CAM
WVHVDSCEQSFDQPYIYSINWNKK
3043.4
3044.0 3048.0 m/z
Expected m/z = 3043.4 Da
WT observed m/z = 3043.4 Da
C191A observed m/z = 3043.4 Da
C124CAM + C129CAM + C132CAM
WCNKPDCNHCGQNTSENMTPLGSQGPNGEESK
3633.7
3633.0 3637.0 3641.0 m/z
Expected m/z = 3633.5 Da
WT observed m/z = 3633.7 Da
C191A observed m/z = 3633.7 Da


e
C191Z-VAD-mk/CAM + C195CAM/Z-VAD-mk
ETRKGRCGEWCNLF
2188.9
WT
C191A
2188.0 2190.0 2192.0 m/z
Expected m/z = 2189.0
Observed m/z = 2188.9
f
C165CAM + C168CAM
IYKCNRCGNITRFPRYNDPIKLLE
3040.6
3040.0 3044.0 3048.0 m/z
Expected m/z = 3040.6 Da
WT observed m/z = 3040.6 Da
C191A observed m/z = 3040.6 Da
C191Z-VAD-mk
ETRKGRCG
1339.7
1340.0 1342.0 1344.0 m/z
Expected m/z = 1339.7 Da
Observed m/z = 1339.7 Da

residues, Cys165, Cys168, Cys191, and the nonconserved residue, Cys195.

To distinguish between these possible sites of modification, z-VAD-fmk-treated WT and C191A were digested with endo Glu-C. This endoprotease principally cleaves on the N-terminal side of glutamate residues. Endo Glu-C was expected to cleave at Glu193, thereby producing fragments containing only Cys191 or Cys195, allowing distinction between modifications on these two residues. Analysis of peptides from the endo Glu-C digest revealed 11 of 14 cysteine residues, including Cys165 and Cys168 (Figure 1f, left panel). We find no evidence for z-VAD-mk modification of either of these residues. A unique peptide of *m/z* 1339.7 Da was observed in endo Glu-C digestion of WT (Figure 1f, right panel). This corresponds to the peptide containing Cys191 bearing z-VAD-mk modification. We do not observe any peptide(s) corresponding to CAM-modified Cys191, nor were we able to identify peptides containing Cys195.

In summary, digestion of WT YPng1 with trypsin, chymotrypsin and endo Glu-C provides a combined 345/363 (95%) sequence coverage, including all 14 cysteine residues and the two Lewis bases of the putative catalytic triad, His218 and Asp235 (see Supplementary information). From these data, we conclude that z-VAD-fmk modification of YPng1 occurs exclusively at the putative active site nucleophile, Cys191. Based on the results presented here, we conclude that z-VAD-fmk, a compound that inhibits PNGase *in vitro* and in living cells, does so by forming a covalent adduct with the putative active site cysteine residue, Cys191. The finding that the His218 is unmodified by z-VAD-fmk is consistent with data observed for caspases, and rules out the possibility of an epoxyketone intermediate. We find no evidence for z-VAD-fmk modification of any other residues, including 13 other cysteines, four of which are absolutely required for proper enzymatic activity.

The preference of PNGase for aspartyl-based inhibitors is noteworthy. Effective recognition and binding of this charged residue suggests that a complementary charged residue, for example, one of the conserved arginine residues of the core domain (Figure 1a), may provide an important binding element within the putative active site.[8] Although z-VAD-fmk is not an obvious mimic of the glycosylated asparagine residues of PNGase substrates, it does present a motif similar to that generated in the products of enzymatic hydrolysis. As such, aspartyl-based peptide inhibitors with small leaving groups, many of which are potent inhibitors of caspases, may likewise inhibit PNGase.

Our data indicate that z-VAD-fmk reacts with PNGase in a manner strictly analogous to its modification of caspases. This result should be taken into account for experiments where the observed effects may be attributed either to inhibition of caspases, inhibition of PNGase or a combination thereof. Such experiments, for example those involving caspase activation and ER stress and that include the use of z-VAD-fmk or related α-halomethylketone inhibitors should therefore be interpreted carefully. An alternative inhibitor, such as Q-VD-OPH,[11] should be used as a pan-caspase inhibitor to avoid such issues since this compound is without effect on PNGase.[9] Nevertheless, given the contribution that peptide sequence of aspartyl fluoromethylketones make to specificity of caspase inactivation, this same class of inhibitor may serve as a scaffold for designing more potent inhibitors of PNGase and caspases that display greater specificity for their intended targets. Such inhibitors will be useful for detailed analysis of the relationships between PNGase inhibition, ER stress, caspase activation and apoptosis.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)

Figure 1 Identification of the site of covalent adduct formation between z-VAD-fmk and PNGase. (**a**) Alignment of conserved amino acids in the core domain of PNGase gene products from various organisms. Sc, *Saccharomyces cerevisiae* (GenBank accession number NP_015229); Hs, Homo sapiens (AAF74720); Mm, *Mus musculus* (AAF74723); Dm, *Drosophila melanogaster* (AAF74722); Ce, *Caenorhabditis elegans* (AAF74721). Residue numbers correspond to the sequence of yeast PNGase. Similarity is identified by yellow, conservation by red. Cysteine residues whose mutation to alanine have been shown to abolish enzymatic activity are indicated by arrows. Residues of the putative catalytic triad are indicated by stars. (**b**) Structures and monoisotopic molecular masses of z-VAD-fmk and the adduct species, z-VAD-mk. (**c**) PNGase was denatured in urea, reduced with DTT, and free cysteines were alkylated with iodoacetamide to afford carboxamidomethyl (CAM) modification. A mass shift of 392 Da, specific to WT PNGase, was observed by MALDI-MS. (**d**) Tryptic digests of z-VAD-fmk treated WT and C191A show putative active site residues His218 and Asp235, as well as conserved residues Cys129 and Cys132, are not the sites of adduct formation. (**e**) Chymotryptic digestion of z-VAD-fmk-treated WT and C191A show Cys191 and Cys195 with modification by one z-VAD-mk and one acetamidomethyl group (CAM). The monoisotopic peak at *m/z* 2187.7 Da in the C191A mutant represents a different chymotryptic peptide species. (**f**) Digestion of z-VAD-fmk-treated WT and C191 with endo Glu-C indicates that adduct formation occurs specifically at Cys191 and not the conserved residues Cys165 or Cys168. Modifications with CAM and z-VAD-mk are denoted by 'CAM' and '-VAD-mk', respectively