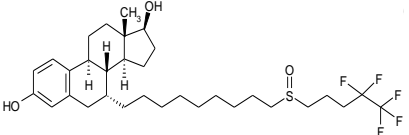
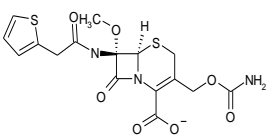
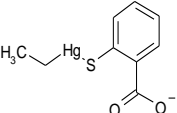
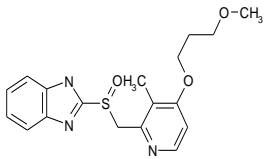
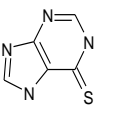
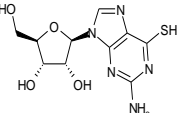
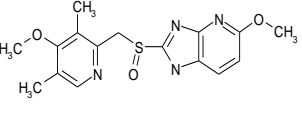
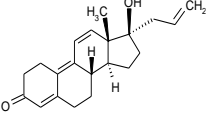
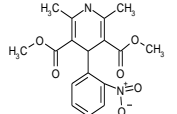
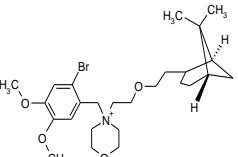
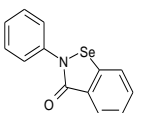
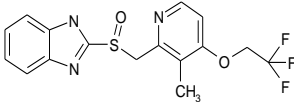
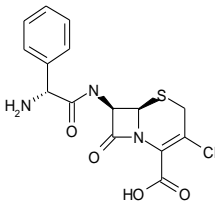
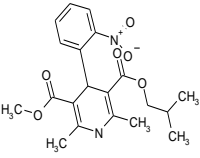
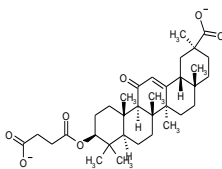


Supplemental data for manuscript # MOLPHARM-AR-2020-000184

Cysteine modification by ebselen reduces the stability and cellular levels of 14-3-3 proteins

Kai Waløen, Jung K.C. Kunwar, Elisa D. Vecchia, Sunil Pandey, Norbert Gasparik, Anne Døskeland, Sudarshan Patil, Rune Kleppe, Jozef Hritz, William H.J. Norton, Aurora Martinez, and Jan Haavik

ID	Drug name	Formula	Therapeutic class/ therapeutic effect	ΔT_m at 400 μM ($^{\circ}\text{C}$)
1	Fulvestrant*		Endocrinology/ Antineoplastic	-23.6
2	Cefoxitin		Metabolism/ Antibacterial	-17.3
3	Thimerosal		Infectiology/ Antiseptic	-11.7
4	Rabeprazole ¹		Metabolism/ Antiulcer	-11.4
5	Mercaptopurine		Immunology/ Immunosuppressant	-10.8
6	Thioguanosine		Metabolism/ Antineoplastic	-9.2
7	Tenatoprazole ¹		Metabolism/ Antiulcer	-8.6
8	Altrenogest*		Endocrinology/ Progestogen	-8.4
9	Nifedipine ²		Cardiovascular/ Antianginal	-7.2
10	Pinaverium ²		Neuromuscular/ Antispastic	-6.7
11	Ebselen		Metabolism/ Anti-inflammatory	-7.0

12	Lansoprazole ¹		Metabolism/ Antiulcer	-6.0
13	Cefaclor		Infectiology/ Antibacterial	-6.0
14	Nisoldipine ²		Cardiovascular/ Antianginal	-5.8
15	Carbenoxolone ^{*,3}		Metabolism/ Antiulcer	-5.1

Supplemental Table 1. Small-molecule drug hits obtained from the primary DSF screen. The midpoint melting temperature (T_m) of 14-3-3 ζ with 4% DMSO was $61.1 \pm 0.5^\circ\text{C}$, and 15 hits that caused $|\Delta T_m| \geq 10 \times \text{SD}$ (5.1°C), used as a cut-off value, were selected for concentration dependent studies. Based on its consistent concentration-dependent effect and reduced toxicity, ebselen was selected for further studies. *Steroids. ¹Proton pump inhibitor. ²Calcium channel blocker ³Probable calcium channel blocker

```

1 [
1 epsilon -MDDREDLVYQAKLAEQAERYDEMVSMMKAVGMDVELTVEERNLLSVAYKNVIGARRASWRISSIEQKEENKGGEDKL 80
2 sigma --MERASLIQKAKLAEQAERYEDMAAFMKGAVEKGEELSCEERNLLSVAYKNVVGQRAAWRVLSSIEQKSNEEGSEKKG
3 gamma --MVDREQLVQKARLAEQAERYDDMAAMKNVTELNEPLSNEERNLLSVAYKNVVGARRSSWRVSSIEQKTSADGNEKKI
4 eta --MGDREQLLQKARLAEQAERYDDMASAMKAVTELNEPLSNEERNLLSVAYKNVVGARRSSWRVSSIEQKTMADGNEKKL
5 theta --MEKTELIQKAKLAEQAERYDDMATCMKAVTEQGAELSNEERNLLSVAYKNVVGARRSARVSSIEQKT--DTSDKKL
6 beta MTMDKSELVQKAKLAEQAERYDDMAAMKAVTEQGHLSNEERNLLSVAYKNVVGARRSSWRVSSIEQKT--ERNEKKKQ
7 zeta --MDKNELVQKAKLAEQAERYDDMAACMKSVTEQGAELSNEERNLLSVAYKNVVGARRSSWRVSSIEQKT--EGAEEKKQ

1
1 epsilon KMIREYRQMVETELKLI CCD I LDVLDKHLI PAAN--TGESKVFYKMKGDYHRYLAEPATGNDRKEAAENSLVAYKAASD 160
2 sigma PEVREYREKVTETELQGVCDTVLGLLDSHLIKEAG--DAESRVFYLKMKGDYRYLAEVATGDDKKRIIDSARSAYQEAAMD
3 gamma EMVRAYREKIEKELEAVCQDVLSLLDNYLIKNCSETQYESKVFYKMKGDYRYLAEVATGEKRATVVSSEKAYSEAFE
4 eta EKVKAYREKIEKELETVCNDVLSLLDKFLIKNCNDFQYESKVFYKMKGDYRYLAEVASGEKNSVVEASEAAYKEAFE
5 theta QL IKDYREKVESELRSI CTTVLELLDKYLI ANAT--NPESKVFYKMKGDYFRYLAEVACGDDRRQTIDNSQGAYQEAFFD
6 beta QMGKEYREKIEAELQDI CNDVLELLDKYLI PNAT--QPESKVFYKMKGDYFRYLAEVASGDNKQTTVSNSSQAYQEAFFD
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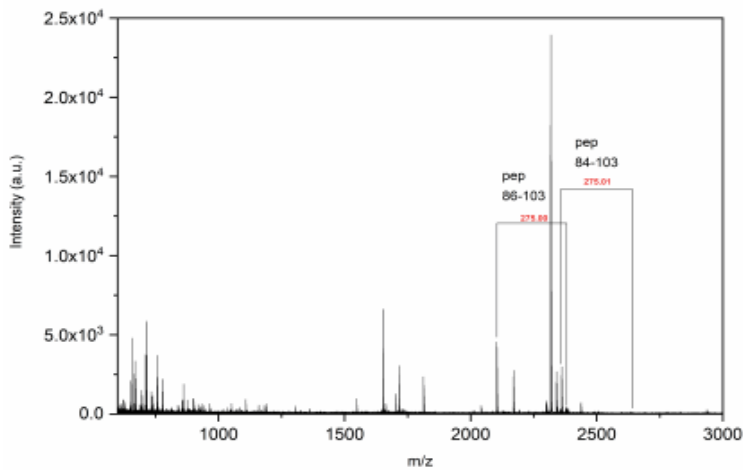
1
1 epsilon IAMTELPPTHPIRLGLALNFSVFYIEILNSPDRACRLAKAAFDDAIAELDTLSEESYKDSLIMQLLRDNLTLWTSDMQG 240
2 sigma ISKKEPPTNPIRLGLALNFSVFHYEIANSPEEAISLAKTTFDEAMADLHTLSEDSYKDSLIMQLLRDNLTLWTADNAG
3 gamma ISKKEHQPTHPIRLGLALNFSVFYIEIQNAPEQACHLAKTAFDDAIAELDTLNEDSYKDSLIMQLLRDNLTLWTSQQD
4 eta ISKKEQMPTHPIRLGLALNFSVFYIEIQNAPEQACHLAKQAFDDAIAELDTLNEDSYKDSLIMQLLRDNLTLWTSQQD
5 theta ISKKEQMPTHPIRLGLALNFSVFYIEILNPELACTLAKTAFDEAIAELDTLNEDSYKDSLIMQLLRDNLTLWTSDSAG
6 beta ISKKEQMPTHPIRLGLALNFSVFYIEILNSPEKACSLAKTAFDEAIAELDTLNEDSYKDSLIMQLLRDNLTLWTSENQG
7 zeta ISKKEQMPTHPIRLGLALNFSVFYIEILNSPEKACSLAKTAFDEAIAELDTLSEESYKDSLIMQLLRDNLTLWTSDTQG

1
1 epsilon DGEEQNKEALQDVEDENQ
2 sigma EEGGEAPQEPQS-----
3 gamma DDGGEGNN-----
4 eta EEAGEGN-----
5 theta EECDAAEGAEN-----
6 beta DEGDAGEGEN-----+
7 zeta DEAEAGEGGEN-----

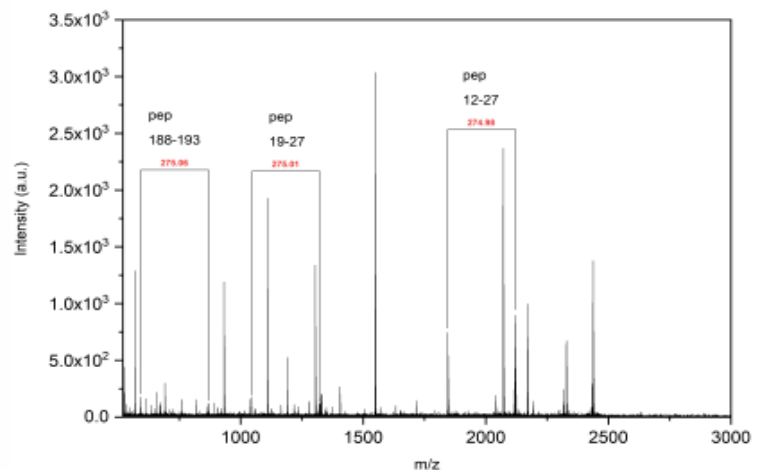
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Supplemental Figure S1. Amino acid sequences of the seven isoforms of 14-3-3 are presented. The cysteine residues are highlighted in yellow.

A 14-3-3ζ C25A-C189A Trypsinated



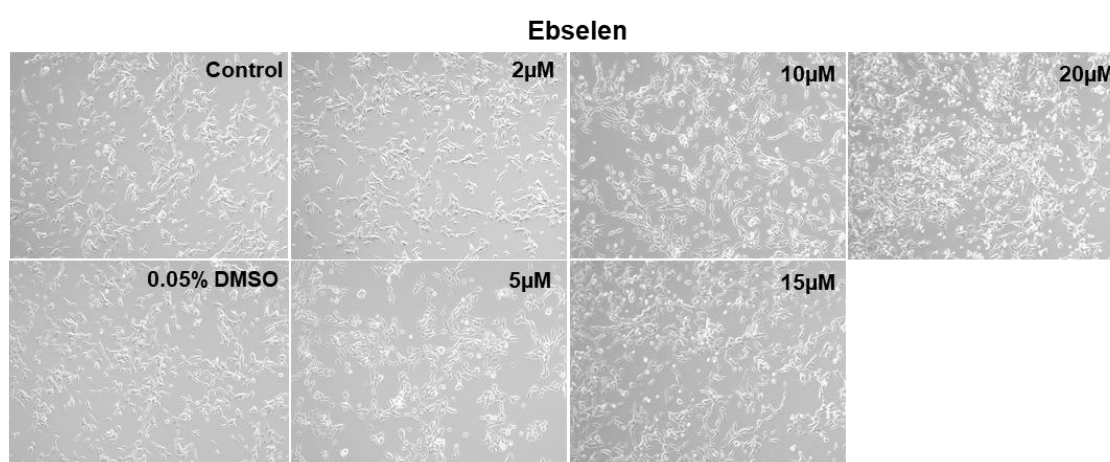
B 14-3-3ζ C94A Trypsinated



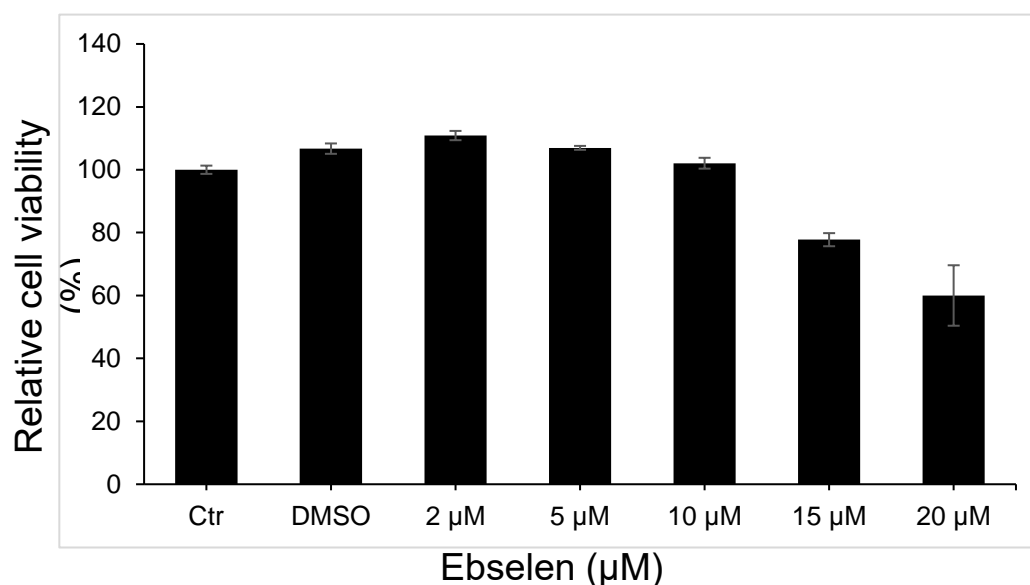
Supplemental Figure S2. MALDI-TOF MS/MS peptide analysis of tryptic digested 1433ζ mutant constructs. a) 1433ζ-C25A-C189A and b) 1433ζ-C94A, after ebselen treatment. Peptides containing cysteines in tagged and untagged form (peptides with a mass shift of 275 Da) are denoted. In a) the figure shows two peptides involving ebselen tagging of C94 in very small amounts. In b) the figure shows a peptide involving C189 and two peptides involving C25.

Supplementary Figure S3. Viability of SHSY5Y cells treated with increasing concentration of ebselen and ebselen oxide. A Cell Titer-Blue assay was performed to examine cell viability of SHSY5Y cells treated with increasing concentration of ebselen and ebselen oxide. The cells were plated in 96-well plates at the density of 25000 cells per well and 5 h later were treated with different concentration of ebselen, ebselen oxide and DMSO (0.05%). Cells were then incubated for 16 h and then 20 μ L of Cell Titer Blue reagent was added to each well. The plates were incubated for 2hrs before the fluorescence ($\lambda=590$) was measured with Victor 3 1420 Multilabel counter plate reader. A, Cell images using ebselen, B, relative cell viability using ebselen, C Cell images using ebselen oxide D, relative cell viability with ebselen oxide. Statistical analyses by 2-tailed Student's t test. (*) $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$, (****) $p < 0.0001$. All data are presented as mean \pm SEM.

A

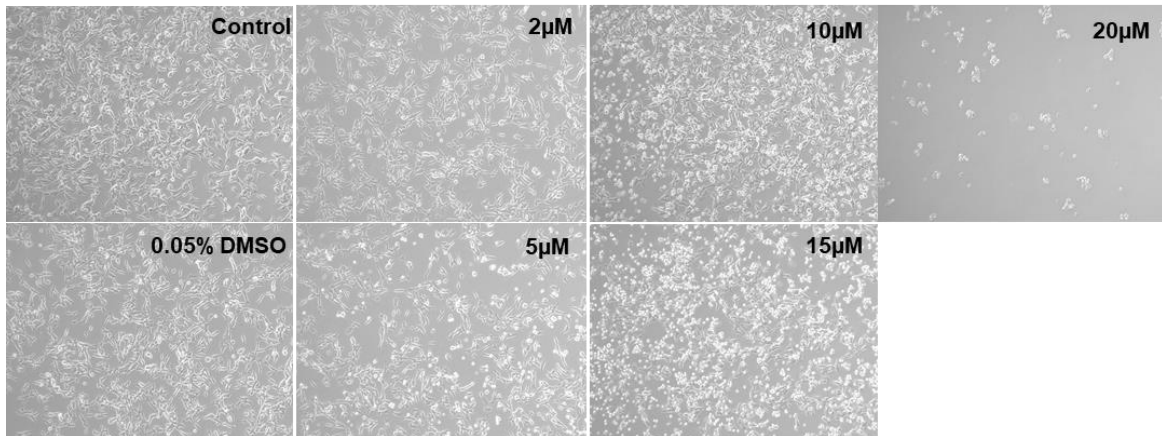


B



C

Ebselen oxide



D

