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VIP **Very Important Paper**

Structure-Activity Studies Reveal Scope for Optimisation of Ebselen-Type Inhibition of SARS-CoV-2 Main Protease

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The reactive organoselenium compound ebselen is being investigated for treatment of coronavirus disease 2019 (COVID-19) and other diseases. We report structure-activity studies on sulfur analogues of ebselen with the Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) main protease (M^{pro}), employing turnover and protein-observed mass spectrometry-based assays. The results reveal scope for optimisation of ebselen/ebselen derivative- mediated inhibition of M^{pro}, particularly with respect to improved selectivity.
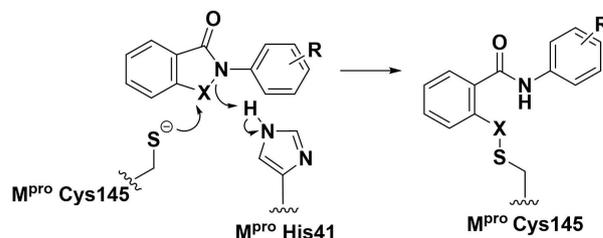
Figure 1. Initial reaction of ebselen/ebsulfur derivatives with the cysteine protease M^{pro}. X = S or Se. Ebselen, X = Se, R = H. Note reaction may also occur with non-catalytic cysteines - M^{pro} has a total of 12 cysteine residues.[7]

Ebselen is a synthetic organoselenium compound which has been under investigation for the treatment of diseases including stroke, hearing loss, and neurodegenerative disorders.[1–3] Ebselen has also been proposed as a treatment for bacterial, viral, and fungal infections.[4–6] Recently, ebselen has attracted attention because of its potential to treat COVID-19, as shown in cell-based assays and by its ability to inhibit the SARS-CoV-2 main protease (M^{pro}), the action of which is essential for the virus life cycle.[7] Ebselen is a potent inhibitor of M^{pro}, with an IC_{50} of < 100 nM.[7] It is proposed to inhibit M^{pro} via covalent reaction of its N–Se bond, in particular with the M^{pro} nucleophilic active site cysteine (Cys145).

Crystallographic studies on the inhibition of catalytic cysteine enzymes, such as Ldt_{Mt2}, by ebselen reveal that an active site nucleophilic cysteine can react with the inhibitor N–Se group to form a catalytically inactive thioselenide, without other fragmentation of the inhibitor.[8] As M^{pro} has a catalytic cysteine, it is possible that a similar process is responsible for the inhibition of M^{pro} (Figure 1). However, a crystal structure with intact ebselen bound to M^{pro} has not been reported and there is crystallographic evidence that ebselen can react with M^{pro} to transfer a selenium atom to the active site cysteine.[9] Mass spectrometric (MS) studies indicate that ebselen can react with multiple nucleophilic residues on M^{pro}.[10] The reactivity of the N–Se ebselen bond is likely important in all its reported biological activities, raising questions about its selectivity and potential toxicity.[11–13]

Here we report initial structure-activity relationship (SAR) studies on the inhibition of M^{pro} by derivatives of ebselen with a sulfur substituted for the selenium (ebsulfur compounds).[14] The MS analyses reveal ebsulfur reacts more than once with M^{pro}, but that some ebsulfur derivatives retain potency against M^{pro} while showing reduced rates of reaction with non-active site nucleophilic residues. Together with other studies reported during the course of our work,[14–15] the results reveal the potential for improvement of ebselen-type inhibition of M^{pro}, perhaps most importantly in terms of selectivity.

The ebsulfur derivatives were synthesised with the aim of investigating their potency and selectivity for the active site Cys145 of M^{pro}. The changes made to ebselen included replacing the selenium for a sulfur atom to decrease reactivity, varying the N-linked sidechain, and adding substituents at the C6 and C8 positions (see Table 1) to alter the fit at the M^{pro} active site. The ebsulfur analogues were synthesised (Figure 2) via amide formation between the appropriate acyl chloride and primary amine to give amides 13–29. Subsequent copper-mediated oxidative sulfur insertion and ring closure as described by Bhakuni et al.[16] gave the cyclised products 3a–c and 3e–11. The bromide substituent of 3c enabled functionalization by Suzuki-Miyaura cross-coupling to give 3d. In one case cyclisation of 29 gave the expected product 12i as well as the oxidised product 12, after purification.

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Table 1. Ebsulfur derivative inhibition and reactivity with M^pro via FRET and SPE-MS assay. See Supporting Information for assay conditions. Note the extent and the number of covalent reactions is likely condition/concentration dependent. See figures S1, S2 and S3 for dose response curves and corresponding covalent modification studies.

Compound	Structure	Apparent max. no. of reactions / most dominant no. of reactions	FRET IC$_{50}$ (µM) / pIC$_{50}$	SPE-MS IC$_{50}$ (µM) / pIC$_{50}$	FRET pIC$_{50}$ / apparent max. no. of reactions
1		12/12	0.04 ± 0.01/ 7.40 ± 0.03	0.26 ± 0.02/ 7.42 ± 0.15	0.6
2		6/2	1.51 ± 0.09/ 5.82 ± 0.03	0.99 ± 0.15/ 6.00 ± 0.06	1.0
3a		11/11	0.13 ± 0.04/ 6.89 ± 0.14	0.34 ± 0.06/ 6.47 ± 0.07	0.6
3b		1/0	>100	>100	N/A
3c		0	>100	10.88 ± 0.82/ 6.28 ± 0.04	N/A
3d		0	>100	>100	N/A
3e		2/1	0.03 ± 0.01/ 7.51 ± 0.10	0.34 ± 0.01/ 6.47 ± 0.02	3.8
3f		9/5	0.48 ± 0.09/ 6.32 ± 0.08	0.43 ± 0.01/ 6.37 ± 0.01	0.7
4a		6/4	0.32 ± 0.04/ 6.50 ± 0.06	0.42 ± 0.03/ 6.38 ± 0.04	1.1
4b		0	>100	>100	N/A
4c		0	94.5 ± 8.22/ 4.03 ± 0.04	>100	N/A
4d		2/0	0.21 ± 0.13/ 6.68 ± 0.08	0.43 ± 0.01/ 6.47 ± 0.02	3.3
5		6/4	0.19 ± 0.04/ 6.75 ± 0.09	0.43 ± 0.03/ 6.36 ± 0.03	1.1
6		6/4	1.00 ± 0.5/ 6.03 ± 0.23	0.53 ± 0.05/ 6.28 ± 0.04	1.0
7		5/4	0.41 ± 0.12/ 6.39 ± 0.13	0.47 ± 0.00/ 6.33 ± 0.00	1.3
8		6/2	0.22 ± 0.10/ 6.67±0.21	0.79 ± 0.09/ 6.10 ± 0.05	1.1
9		3/1	0.51±0.02/ 7.51 ± 0.10	2.55 ± 0.57/ 5.60 ± 0.1	2.1
10		5/2	0.95 ± 0.04/ 6.11 ± 0.10	0.78 ± 0.18/ 7.51 ± 0.10	1.2
11		6/2	0.25 ± 0.02/ 6.61 ± 0.04	0.39 ± 0.05/ 6.41 ± 0.05	1.1
12		0	2.83 ± 0.34/ 5.55 ± 0.05	20.12 ± 1.12/ 4.70 ± 0.02	N/A

Figure 2. Synthesis of ebsulfur derivatives **3a–f**, **4a–d**, **5–12**. Reagents and conditions: **A**: oxalyl chloride, CH$_2$Cl$_2$, DMF, 0°C→rt. **B**: Et$_3$N, R$_3$NH$_2$, CH$_2$Cl$_2$, rt. **C**: CuI, 1,10-phenanthroline, S$_8$, K$_2$CO$_3$, DMF, 110°C. **D**: phenylboronic acid, K$_2$CO$_3$, Pd(dppf)Cl$_2$, 1,4-dioxane, 80°C.

the catalytic Cys145 of M^pro by protein-observed mass spectrometry (POMS) under denaturing conditions.[10] M^pro contains a total of twelve cysteines,[7] hence the number of covalent reactions observed with M^pro gives potential insight into the degree of active site selectivity of the ebsulfur compounds.

The turnover assay results (Table 1) show that the direct ebsulfur analogue (**3a**) of ebselen (**1**) has a comparable IC$_{50}$ to ebselen (**1**), confirming that an S–N bond is sufficiently reactive to inhibit M^pro at nanomolar concentrations. The unsubstituted ebsulfur benzoisothiazolinone (BIT) core (**2**) was much less active (IC$_{50}$ ~ 1.6 µM). Ebsulfur derivatives **3a**, **4a**, **5–7**, varying solely in the *N*-linked hydrophobic sidechain, were synthesised since it was envisaged that these sidechains might occupy the same binding pocket as the sidechain of the P1′ residue of M^pro substrates, as anticipated based on the structures of substrate-like inhibitors.[7] These compounds were the most potent M^pro inhibitors described here, i.e. **3a** (*N*-phenyl) ~0.13 µM; **4a** (isopentyl) ~0.32 µM; **5** (*N*-benzyl) ~0.19 µM; **6** (*N*-cyclohexylmethyl) ~1.00 µM; and **7** (n-pentyl) ~0.41 µM. The variations in IC$_{50}$s for the compounds, together with the results of Sun *et al.*[14] show clear scope for further optimisation of the *N*-substituent of the ebsulfur derivatives (see supplementary Figures S1 and S2).

The ebsulfur compounds were initially screened for M^pro inhibition activity using a fluorescence-based turnover (FRET) assay and a solid-phase extraction coupled to mass spectrometry (SPE-MS) assay, and for selectivity in terms of reaction with

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Figure 3. Dose response curves obtained using FRET assay and protein observed mass spectrometry. **A**) 1 (Ebselen) inhibits M^{pro} and reacts at multiple sites. **B**) 4d inhibits M^{pro}, but reacts covalently at less sites than ebselen. **C**) 4c (an isomer of 4d) apparently does not inhibit or react with M^{pro}. **D**) 3e manifests good inhibition and apparently reacts twice, but predominantly once. **E**) 3c apparently does not inhibit or react with M^{pro}. **F**) 3d shows no inhibition and does not react with M^{pro}, potentially due to steric hindrance by its C-8 phenyl group. **G**) 11 inhibits and reacts. **H**) 4a inhibits and reacts, predominantly 4 times. **I**) 9 inhibits M^{pro} and apparently reacts with up to 3 times, but predominantly once. Conditions: 40 μM compound, 2 μM M^{pro}, 20 mM HEPES, pH 7.5, 50 mM NaCl. ([a]) and ([b]) represent technical duplicates for POMS studies. See Supporting Information for assay conditions.

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Several compounds with more polar *N*-substituents (**8**–**11**) were synthesised to increase solubility and/or selectivity, including **9**, the γ-lactam of which was envisaged may bind in an analogous manner to the P1 glutamine sidechain in M^{pro} substrates.[7] None of these compounds were as potent as **1** or **3a**, perhaps reflecting the hydrophobic nature of regions of the M^{pro} active site, including part of the P1 residue binding sub-site.[7]

Increasing the oxidation state of the sulfur substantially increased the IC$_{50}$ (**12**, ~2.9 μM cf. **11** 0.25 μM), suggesting steric hindrance may be one factor in determining reactivity (Figure 3). We thus made **3b**–**d**, **4b,c** with methyl-, phenyl-, or bromo-derivatives at the C8 position.

Such derivatisation substantially reduced potency: **4c** has an IC$_{50}$ of ~94.5 μM, and **3b**–**d**, and **4b** have IC$_{50}$s > 100 μM. Conversely, C6-substitution had the opposite effect; addition of a bromine at the C6 position of **3a** and **4a**, to give **3e** and **4d**, improved potency relative to values for the unsubstituted compounds (**3e**, ~0.03 μM cf. **3a** ~0.13 μM, and **4d**, ~0.21 μM cf. **4a**, ~0.32 μM). Introduction of a nitrogen at the C6 position to give the pyridine (**3f**) showed a slight decrease in potency (IC$_{50}$ ~0.5 μM).

It is important to note that it cannot be assumed that inhibition is mediated via reaction with Cys145 alone and derivatisation may alter the patterns of reactivity with the 12 M^{pro} cysteines. We thus carried out protein-observed mass spectrometry (MS) under denaturing conditions to compare the number of (irreversible) covalent reactions by the ebsulfur derivatives. Although we did not carry out time-course studies (except for ebselen and ebsulfur[10]), and the nature and the extent of the reactions will likely vary depending on the conditions, the results give initial insight into the relative reactivity of the compounds.

The results show that there is a general, but clearly imperfect, correlation between the apparent extent of covalent reaction and potency, consistent with the proposed mode of reaction of the ebsulfur derivatives with cysteines to form disulfide bonds (Figure 1). However, it should be noted that alternative modes of covalent reaction are possible, e.g. via reaction of the ebsulfur derivative carbonyl group. Secondly, introduction of substituents at the C6 and C8 positions of ebsulfur decreases the number of reactions. Thus the presence of bromo- or phenyl- substituents at C8 apparently completely prevents irreversible covalent reaction (**3c**, **3d**, **4c**). C8 methyl substitution shows a reduction in the apparent maximum number of observed reactions from 6 (**4a**) to 0 (**4b**) for the *N*-isopentyl sidechain, and from 11 (**3a**) to 1 (**3b**) for the *N*-phenyl sidechain; increasing the oxidation state of the sulfur also apparently eliminates observed covalent reaction (**12**). Importantly, the presence of a more polar *N*-substituent (compare **9**, **11**/1 and 2 reactions, with **6**, **7**/both 4 reactions) or C6 bromine (compare **3e**/1 reaction with **3a**/11 reactions, and **4d**/no reaction with **4a**/4 reactions) reduces the dominant number of observed reactions. The combined IC$_{50}$ and MS results for **9**, **4d**, and, particularly, for **3e**, are notable as they suggest that optimised substitution of the ebsulfur core to achieve enhanced selectivity in terms of its reaction with M^{pro} should be possible.

We suggest that optimising the pIC$_{50}$/number of reactions observed by MS as a useful, readily obtainable parameter for initial optimisation of reaction selectivity (Table 1).

The reactivity of ebselen or ebselen/ebsulfur derivatives with cysteines, and maybe other biomolecules, means that there are likely substantial challenges in developing these compounds as medicines. While the activities of the ebsulfur compounds reported here do not improve on the potency of ebselen, they clearly demonstrate the potential for achieving compounds that are more selective in their reaction with M^{pro} and, by implication, other biomolecules including both proteins and small-molecule thiols such as glutathione/cysteine. This is clearly exemplified by comparing the MS results for ebselen, which, under our standard conditions, covalently reacts up to 12 times with M^{pro}, with those for **3e**, which was observed to only react twice under our conditions, yet retains substantial potency (~0.03 μM).

Under our conditions, we saw no evidence for the fragmentation of the ebsulfur derivatives or ebselen, as has been provided on the basis of crystallographic and other evidence for ebselen itself,[9] potentially in part reflecting differences in reaction conditions/methods of analysis. We suggest that if such improvements in selectivity can be achieved by simple sulfur for selenium substitution, then there is considerable scope for more sophisticated 'caging' of the reactive N–Se/S bonds of ebselen/ebsulfur type compounds. Induced-fit binding to M^{pro} or another target could result in exposure of a reactive N–S/Se bond, which is otherwise protected from nucleophilic attack by other endogenous cysteines. It is also likely the potency and selectivity of the compounds can be improved by taking advantage of inter-actions observed with other types of M^{pro} inhibitor, e.g. incorporation of a benzisothiazolinone core into a larger structure that mimics the binding of the natural substrates.

Conflict of Interest

The authors declare no conflict of interest.

[1] G. K. Azad, R. S. Tomar, *Mol. Biol. Rep.* **2014**, *41*(8), 4865–4879.

[2] M. J. Parnham, H. Sies, *Biochem. Pharmacol.* **2013**, *86*(9), 1248–1253.

[3] J. Kil, E. Lobarinas, C. Spankovich, S. K. Griffiths, P. J. Antonelli, E. D. Lynch, C. G. Le Prell, *Lancet* **2017**, *390*(10098), 969–979.

[4] H. Sies, M. J. Parnham, *Free Radical Biol. Med.* **2020**, *156*, 107–112.

[5] S. Thangamani, W. Younis, M. N. Seleem, *Sci. Rep.* **2015**, *5*, 11596.

[6] S. Thangamani, H. E. Eldesouky, H. Mohammad, P. E. Pascuzzi, L. Avramova, T. R. Hazbun, M. N. Seleem, *Biochim. Biophys. Acta Gen. Subj.* **2017**, *1861*(1), 3002–3010.

[7] Z. Jin, X. Du, Y. Xu, Y. Deng, M. Liu, Y. Zhao, B. Zhang, X. Li, L. Zhang, C. Peng, Y. Duan, J. Yu, L. Wang, K. Yang, F. Liu, R. Jiang, X. Yang, T. You, X. Liu, X. Yang, F. Bai, H. Liu, X. Liu, L. W. Guddat, W. Xu, G. Xiao, C. Qin, Z. Shi, H. Jiang, Z. Rao, H. Yang, *Natur* **2020**, *582*(7811), 289–293.

[8] M. de Munnik, C. T. Lohans, P. A. Lang, G. W. Langley, T. R. Malla, A. Tumber, C. J. Schofield, J. Brem, *Chem. Commun.* **2019**, *55*(69), 10214–10217.

[9] K. Amporndanai, X. Meng, W. Shang, Z. Jin, M. Rogers, Y. Zhao, Z. Rao, Z.-J. Liu, H. Yang, L. Zhang, P. M. O'Neill, S. Samar Hasnain, *Nat. Commun.* **2021**, *12*(1), 3061.

[10] T. R. Malla, A. Tumber, T. John, L. Brewitz, C. Strain-Damerell, C. D. Owen, P. Lukacik, H. T. H. Chan, P. Maheswaran, E. Salah, F. Duarte, H. Yang, Z. Rao, M. A. Walsh, C. J. Schofield, *Chem. Commun.* **2021**, *57*(12), 1430–1433.

[11] F. C. Meotti, V. C. Borges, G. Zeni, J. B. T. Rocha, C. W. Nogueira, *Toxicol. Lett.* **2003**, *143*(1), 9–16.

[12] C. Jacob, W. Maret, B. L. Vallee, *Biochem. Biophys. Res. Commun.* **1998**, *248*(3), 569–573.

[13] R. Sekirnik, N. R. Rose, A. Thalhammer, P. T. Seden, J. Mecinović, C. J. Schofield, *Chem. Commun.* **2009**, 42, 6376–6378.

[14] L.-Y. Sun, C. Chen, J. Su, J.-Q. Li, Z. Jiang, H. Gao, J.-Z. Chigan, H.-H. Ding, L. Zhai, K.-W. Yang, *Bioorg. Chem.* **2021**, *112*, 104889.

[15] M. Zmudzinski, W. Rut, K. Olech, J. Granda, M. Giurg, M. Burda-Grabowska, L. Zhang, X. Sun, Z. Lv, D. Nayak, M. Kesik-Brodacka, S. K. Olsen, R. Hilgenfeld, M. Drag, *bioRxiv* **2020**. doi: https://doi.org/10.1101/2020.08.30.273979.

[16] B. S. Bhakuni, S. J. Balkrishna, A. Kumar, S. J. T. L. Kumar, *Tetrahedron Lett.* **2012**, *53*(11), 1354–1357.