Diselenolane-mediated cellular uptake†

Nicolas Chuard,a Amalia I. Poblador-Bahamonde,a Lili Zong,a Eline Bartolami,a Jana Hildebrandt,b Wolfgang Weigand, b Naomi Sakaia and Stefan Matile b, a

The emerging power of thiol-mediated uptake with strained disulfides called for a move from sulfur to selenium. We report that according to results with fluorescent model substrates, cellular uptake with 1,2-diselenolanes exceeds uptake with 1,2-dithiolanes and epidithiodiketopiperazines with regard to efficiency as well as intracellular localization. The diselenide analog of lipoic acid performs best. This 1,2-diselenolane delivers fluorophores efficiently to the cytosol of HeLa Kyoto cells, without detectable endosomal capture as with 1,2-dithiolanes or dominant escape into the nucleus as with epidithiodiketopiperazines. Diselenolane-mediated cytosolic delivery is non-toxic (MTT assay), sensitive to temperature but insensitive to inhibitors of endocytosis (chlorpromazine, methyl-b-cyclodextrin, wortmannin, cytochalasin B) and conventional thiol-mediated uptake (Ellman’s reagent), and to serum.

Selenophilicity, the extreme CSeSeC dihedral angle of 0° and the high but different acidity of primary and secondary selenols might all contribute to uptake. Thiol-exchange affinity chromatography is introduced as operational mimic of thiol-mediated uptake that provides, in combination with rate enhancement of DTT oxidation, direct experimental evidence for existence and nature of the involved selenol sulfides.

Unlike simple alkanes or peroxides, the most favorable CXXC dihedral angle \( \theta \) in acyclic diselenides 1 and disulfides 2 is \( \sim 90° \) (\( X = S, Se \), Fig. 1).1 Decreasing \( \theta \) in cyclic disulfides causes the increase of ring tension in 1,2-dithianes with 62° over 1,2-dithiolanes 3–4 with 27–35° to approach the maximum epidithiodiketopiperazine (ETP) 5 with \( \sim 0° \).2 Earlier, we found the dependence of the ability of cyclic disulfides as delivery vehicles of otherwise cell-impermeable cargos to the ring tension.1,2 Mechanistic studies support that the ring tension promotes dynamic covalent thiol-disulfide exchange on the cell surface1,3 to initiate uptake.1,2 Asparagusic acid (SA) derivative 4 efficiently delivers functional peptides and objects as large as intact liposomes.6

This emerging power of strain-promoted thiol-mediated uptake with cyclic disulfides called for cyclic diselenolanes. Besides many similarities, disulfides and selenides have also important differences. In six-membered rings, a very similar \( \theta \) of \( \sim 60° \) (56°) has been confirmed.7 In the crystal structure of 1,2-diselenolanes like Sea 6, the CSeSeC dihedral angle is 0.2°, contributing to an almost ideal, twist-free envelope structure of the five-membered ring.8,9 At the same time, the Se–Se bond

---

*Department of Organic Chemistry, University of Geneva, Geneva, Switzerland. E-mail: stefan.matile@unige.ch; Web: http://www.unige.ch/sclences/chiorg/matile/; Tel: +41 22 379 6523

†Electronic supplementary information (ESI) available: Detailed procedures and results for all reported experiments. See DOI: 10.1039/c7sc05151d

---

Fig. 1  (a) Selected CXXC dihedral angles, XX bond length and absorption maxima of disulfides and diselenides, and the intrinsic \( pK_a \) of one thiol or selenol in their reduced form (\( X = S, Se; R, R', R'', \), see Fig. 2). (b) B3LYP/6-311++G** energies of MeXXMe conformers. (c) Minimized structure of the parent 1,2-dioxo- (O = red), dithio- (S = yellow) and diselenolane (Se = orange) with angles (CXXC, CXX) and bond length (XX), arrows: endo position.
length increases from ~2.3 Å in relaxed diselenides 1 to 2.38 Å in 1,2-diselenolane 6, and the absorption maximum shifts from 320 nm to 430 nm. The CSSC dihedrals of 1,2-dithiolanes maximize at 27° in SAs such as 4, and absorptions shift from 250 nm to maximal 340 nm. The crystal structures of 1,2-di-selenolanes show layers of selenium with chalcogen bonds of down to 3.55 Å length between the polarizable selenium atoms of different molecules. In the solid state, the strained di-selenolanes easily polymerize into a gum. In solution, relaxed diselenides exchange up to 7 orders of magnitude faster than disulfides. The high acidity of selenols (pK_a ~ 5.2) compared to thiols (pK_a ~ 8.2) contributes to this increased reactivity by activating both nucleophile and leaving group for selenol-diselenide exchange in neutral and also slightly acidic water. However, although exchange is possible with both thiols and selenols, the formation of Se-Se bonds is favored over mixed S-Se bonds. This selenophilicity has been used elegantly to catalyze protein folding and protein denaturation with di-selenides in the presence of immobilized thiols.

Some of the characteristics of 1,2-diselenolanes are reminiscent of ETPs (i.e., pK_a < 7, CXXC dihedral ~0°, Fig. 1) and could thus promise similarly outstanding uptake efficiencies with dual activation at highest tension. While to note in this context is also the presence of eight out of the 25 human selenoproteins in cellular membranes, which could potentially participate in selenol-diselenide exchange reactions during the uptake of diselenides. Moreover, cellular uptake, particularly gene transfection, with tension-free poly(diselenide)s and poly(ditelluride)s has been reported to occur and differ sometimes favorably from uptake with poly(disulfide)s. In the following, we report uptake of 1,2-diselenolanes SeL 7 and SeA 6 to the cytosol without endosomal capture as with the corresponding 1,2-dithiolanes SL 3 and SA 4 or dominant escape into the nucleus as with ETP 5.

To elaborate on diselenolane-mediated uptake, green-fluorescent fluorescein isothiocyanate (FITC) conjugates were synthesized. SeL 7 was prepared from dichloride 8 (Fig. 2 and Scheme S1†). The reaction with Na_2Se_2 afforded the 1,2-di-selenolane scaffold as in the previously reported diseleno lipidic acid 9. Activation with N-hydroxysuccinimide (NHS) afforded the “SeL tag” 10, an activated ester that reacted readily with amines such as FITC-amine 11 to afford SeL 7. The green-fluorescent diselenolane SeA 6 was prepared from diseleno asparagusic acid following the same route (Scheme S2†), and the acyclic diselenide 1 was obtained by reaction of methylated diseleno cystine (SeC) with FITC (Scheme S3†). The control compounds SA 4 and ETP 5 were prepared following previously reported procedures.

In computational studies, the minima at 90° and maxima at 0° in the conformational profiles of MeSeSeMe and MeSSMe were confirmed, whereas MeOOMe naturally minimized in anti conformation (Fig. 1b). The decreasing energy of the maxima from O to S and Se at 0° supported that ring tension in cyclic diselenides is lower than in disulfides. For unsubstituted 1,2-dioxo-, dithio- and diselenolanes, this trend resulted in envelope conformers with the chalcogen atom, the α carbon, and the β carbon in endo position and CXX angles decreasing from 102° to 95° and 91°, respectively (arrows, Fig. 1).

With tris[2-carboxyethyl]phosphine (TCEP), 1,2-diselenolane 9 was readily reduced to diseleno SeL(R) 12' (Fig. 2). However, neither SeA nor SeL appeared to react with various thiols 13 (Fig. 3). These results implied that either diselenides do not undergo exchange reactions with thiolates or, unlike S-S homologs 14, the selenosulfide intermediate products 15–17 ring close easily by intramolecular selenol–selenosulfide exchange. Their existence and nature was thus explored by thiol-exchange affinity chromatography (Fig. 3). Compared to a non-reactive carboxylfluorescein (CF) standard (Fig. 3a), all disulfides and diselenides showed delayed elution as expected.

---

Fig. 2 Complete structure of all transporters used (neutral form), with representative synthesis and reduction for SeL 7. (a) (1) Several steps, ref. 17; (2) NHS, DCC, THF, rt, 18 h, 53%; (b) several steps, ref. 1; (c) DMF, rt, 3 h, 14%; (d) TCEP, <5 min; (e) air.
for temporal covalent bonding with the solid phase through thiol phase (Fig. 3b–f, solid). Although the retention time of these peaks was shortened a little (~1 min) in the presence of DTT in the mobile phase (Fig. 3b–f, dashed), these retentions were supported but did not prove transient dynamic covalent bonding to the solid phase.

With SA 4, a second broad peak eluted only upon addition of DTT to the mobile phase (Fig. 3c, *). This observation confirmed the formation and stability of the ring-opened, strain-released exchange product 14 by covalent attachment to the stationary phase. The absence of this second peak with SeA 6 indicated that ring-opened S-Se product 15, if formed on the exchange column, ring closes rapidly back to SeA 6 (Fig. 3d). This result was consistent with expectations from the reduced ring tension in 1,2-diselenolanes compared to 1,2-dithioles, selenophlicity and the low pK\textsubscript{a} of the selenol (Fig. 1). The importance of this low pK\textsubscript{a} was confirmed by the absence of this second DTT-dependent peak also for ETP 5 with thiols of comparable acidity (Fig. 1 and 3b).

With SeL 7, a second broad peak could be observed upon addition of DTT to the mobile phase (Fig. 3e, *). This finding provided direct experimental evidence for the occurrence of thiol-diselenide exchange with SeL 7, i.e., existence of the ring-opened S-Se product 16. This S-Se product 16 and not the constitutional isomer 17 was presumably formed first because a primary selenolate with a lower pK\textsubscript{a} is a better leaving group than the secondary selenolate with a higher pK\textsubscript{a}. However, S-Se isomer 16 was expected to exchange intramolecularly because the secondary selenol in S-Se isomer 17 with a higher pK\textsubscript{a} is a weaker nucleophile. The reduced reactivity of this S-Se isomer 17 with a secondary selenol could explain the detectability of the second, DTT-dependent peak in the affinity chromatogram of SeL 7 but not for SA 6 with primary selenols only (Fig. 3e, *). The isomerization from more reactive 16 to less reactive 17 could also account for the split of the first-eluting peak into two, a behavior that was unique for SeL 7 (Fig. 3e). An intense, very broad, DTT-dependent second peak was also observed with the acyclic diselenol cystine SeC 1 (Fig. 1 and 3f, *). The appearance of this peak was as expected because of the absence of a nearby selenol for rapid release by intramolecular exchange.

In the permanent presence of 50 mM DTT in the mobile phase, the fast eluting double peak of SeL 7 at R\textsubscript{t} = 36.1 min was followed by smaller but significant additional peak (Fig. 3e, **). A similar peak occurred also with ETP 5 (Fig. 3b, **). A possible explanation of this observation was that DTT reduces SeL 7 to the diselenol 12 (Fig. 2), which then offers the activated primary selenolate to exchange with “hidden” disulfides 18 on the solid phase to give the more stable selenosulfide 17, which undergoes ring closure or exchanges with DTT slowly enough to appear as a separate peak. The same arguments were applicable to ETP 5. This plausible explanation implied the existence of less reactive disulfides 18 on the solid phase that resist DTT but not the more reactive SeL\textsuperscript{A} 12 and reduced ETP 5. Although speculative, this conclusion caught our full attention because it would be consistent with unique properties of SeL 7 and ETP 5 with regard to cellular uptake (see below).

Further support of the possible thiol-diselenide exchange was obtained by measuring the oxidation kinetics of DTT 19 to DTT\textsuperscript{2+} 20 catalyzed by diselenides.\textsuperscript{15} In the presence of one equivalent seleno lipoic acid 9, the halflife time of DTT 19 decreased from t\textsubscript{50} = 16 h (Fig. 4, ○) to t\textsubscript{50} = 6.9 h (Fig. 4, ■). This rate enhancement confirmed the occurrence of thiol/diselenide exchange, the existence of intermediate 21 with mixed S-Se bonds and fast oxidation of SeL\textsuperscript{A} 12 by molecular oxygen. The catalysis of DTT oxidation by 1,2-diselenolane 9 also demonstrated that the reloading of ring tension in 9 is less
favored than ring closure of DTT into the much less strained DTT
d (Fig. 4, arrows).

Even more significant rate enhancements for DTT oxidation were observed with seleno asparagusic acid (SeA, Fig. 2 and 4, O). Compared to 1,2-disenelenolane 9, DTT lifetimes dropped further from \( t_{0,2} \approx 6.9 \) h to \( t_{0,3} \approx 3.9 \) h in the presence of SeA, and the final second-order rate constant increased from \( k = 1.4 \times 10^{-2} \) M\(^{-1}\) s\(^{-1}\) to \( k = 8.9 \times 10^{-2} \) M\(^{-1}\) s\(^{-1}\). These results provided the experimental evidence for thiol-diselenide exchange for SeA that could not be secured unambiguously by thiol-exchange affinity chromatography (Fig. 3d).

In summary, the passage through thiol-exchange affinity columns complemented by DTT oxidation catalysis reflected pertinent aspects of cellular uptake well (see below). The results demonstrated compatibility of 1,2-diselenolanes with strain-promoted thiol-mediated uptake. Moreover, evidence became available for dynamic covalent exchange with thiols on the cell surface, possible hopping along thiols or disulfides, facile intracellular release, possible participation of less reactive cellular disulfides and thiols, and clear differences between diselenides Sel 7, SeA 6 and SeC 1 as well as disulfides SA 4 and ETP 5.

The quantitative detection of cellular uptake was complicated by the fluorescence quenching with 1,2-diselenolanes. Compared to already strongly quenched 1,2-dithiolane SA 4, 1,2-diselenolanes Sel 7 and SeA 6 displayed even weaker emission of FITC in buffer by factors of 4.6 and 3.3, respectively (Fig. 5b, solid, blue, red vs. green). Adding the 3.4-fold quenching of free FITC by 1,2-dithiolane in 4, the total quenching by 1,2-diselenolane calculated to a factor of up to 15.6. Fluorescence partially recovered upon reduction with TCEP (Fig. 2, 5a and b). Compared to reduced SA 4, correction factors of 1.9 and 1.5 were obtained for Sel 7 12 and reduced form of SeA 6 (Fig. 5b, dashed, blue, red vs. green). For the quantification of uptake experiments, correction factors for closed cycles were applied.

The uptake of the green-fluorescent SA 4, SeA 6 and Sel 7 into HeLa Kyoto cells was monitored by flow cytometry and confocal laser scanning microscopy (CLSM). Flow cytometry analysis with 10 \( \mu \)M of diselenides in Leibovitz medium at 37 °C showed a gradually increasing uptake with time, with 1,2-diselenolanes being much more active than 1,2-dithiolane SA 4, and with Sel 7 outperforming SeA 6 (Fig. 5c).

These impressions were confirmed in CLSM images, taken after one hour of incubation. Dithiolane SA 4 showed the known punctate emission characteristic for substantial capture in endosomes, together with significant homogeneous emission from the cytosol (Fig. 6a). SeA 6 showed stronger and more homogeneous emission at 50 \( \mu \)M but was visibly cytotoxic at 100 \( \mu \)M (Fig. 6b). With Sel 7, intact cells were stained brightly and homogeneously including nuclei, at 50 \( \mu \)M and also at 100 \( \mu \)M (Fig. 6c).

Cytotoxicity was determined with the MTT assay, an assay that reports on the metabolic activity of the cells. Like SA 4 and ETP 5, Sel 7 was not cytotoxic up to concentrations as high as 100 \( \mu \)M (Fig. 7a). As indicated by CLSM images (Fig. 6b), cytotoxicity started to emerge at 100 \( \mu \)M of SeA 6, although...
10 μM CPPs remained clearly more toxic (Fig. 7a). Studies on SeA were thus discontinued.

Diselenolane-mediated uptake of SeL 7 was less sensitive to temperature than ETP-mediated uptake, for instance (Fig. 7b). Insensitivity toward inhibitors supported that SeL does not enter cells by clathrin-mediated endocytosis (chlorpromazine; CPZ), caveolae-mediated endocytosis (methyl-β-cyclodextrin; mβCD) and macropinocytosis (wortmannin; wort, cytochalasin B; cytoB). The presence of 10% serum slightly decelerated uptake without reducing final efficiency significantly. As for ETP 5 but neither for SA 4 nor SL 3, uptake of SeL 7 was insensitive to DTNB, an inhibitor of conventional thiol-mediated uptake. In agreement with results from thiol-exchange affinity columns (Fig. 3), this might suggest that SeL 7 and ETP 5 can exchange also with less reactive thiols on the cell surface that are beyond reach of Ellman’s reagent.

After longer incubation times, SeL 7 was efficiently delivered also at 10 μM (Fig. 8a–c). Z-stacks for SeL 7 confirmed that the homogeneous emission originates from cytosol (Fig. 8e). Corroborative support for delivery to cytosol and nucleus could be seen by the poor emission from nucleoli (Fig. 8d, arrows). Entry into the nucleus but not the nucleoli contrasted nicely to CPDs and other polycationic carriers that can accumulate in nucleoli, presumably due to interactions with polyanionic DNA, and to ETP 5, which targets the nucleus including nucleoli.

The acyclic diseleno cystine SeC 1 showed weaker activity and mostly produced the punctate emission characteristic for endosomal capture (Fig. 8d and 9). This behavior was similar to SA 4 (Fig. 9) and in excellent agreement with mechanistic expectations from thiol exchange affinity columns (Fig. 3). More specifically, the selenosulfides obtained by thiol-diselenolane exchange of SeC 1 on the cell surface are much more stable than with SeL 7 because the proximal covalent selenol for ring closure and release is missing (Fig. 3f). The similarity in affinity chromatogram of the most active ETP 5 and SeL 7, particularly the unique, DTNDb-dependent peak (Fig. 3, **), implied the transient existence of the fully reduced form and its exchange with less reactive cellular disulfides during uptake (12, Fig. 9). For 1,2-diselenolane-mediated uptake, this added up to a putative mechanism that begins with exchange of poorly reactive thiols that is stable enough for detection by affinity chromatography (Fig. 3d*). Somewhere during uptake, thiol exchange with selenosulfide isomer 17 releases SeLR, which exchanges with less reactive cellular disulfides before it can back-oxidize to 7 (interpretation of **peak in affinity chromatogram, Fig. 3). Further exchange with other cellular thiols 13 and disulfides 18 is possible until SeL 7 or SeLR 12 are released into the cytosol (Fig. 4 and 9). The result for diselenolane-mediated cytosolic delivery with SeL 7 is a quite spectacular target hopping mechanism on different oxidation levels that will be extremely difficult to clarify in detail (Fig. 9).
The same conclusions apply to the similarly promising cytosolic delivery with ETP 5.

Several possible additions and alternatives to this putative uptake mechanism exist. For instance, the free selenenyl next to the selenolsulfide in conjugates 16 or 17 could exchange with another exofacial disulfide to produce a second selenolsulfide and afford a more stable conjugate, whereas further dimerization of oligomerization are unlikely due to high dilution and fast ring closure (Fig. 9). Glutathione at the cell surface could be involved and possibly initiate uptake with partially or fully reduced SeL 12,18,12, exchanging first with exofacial disulfides 18, instead of oxidized SeL 7, exchanging first with exofacial thiols 13 (Fig. 9).45–47 Intracellular retention could contribute to uptake efficiencies and should influence intracellular location31 (uptake of underivatized CF was negligible under experimental conditions). These and other uncertainties concerning a complex mode of action should not distract from the key facts disclosed in this report: (a) Cytosolic delivery by SeL 7 is outstanding (Fig. 9).21 (b) Clear differences to SeA 6 (cytotoxic, Fig. 6b and 7a; insensitive to affinity columns, Fig. 3d and e) indicate that a secondary selenol is essential (they very well reflect the distinct differences between SL and SA with regard to cellular uptake1 and polymerization on surfaces23 and in solution24). (c) Clear differences to SeC 1 (endosomal capture, Fig. 9; permanent retention on affinity columns, Fig. 3f and e) indicate that a strained cyclic diselenide is essential. (d) All trends, covering diselenides and disulfides from molecular modeling to affinity chromatography and intracellular localization, are consistent with conceptual expectations.

In summary, the move from sulfur to selenium affords the best carriers identified in this series so far. Cellular uptake mediated by 1,2-diselenolane 7, i.e., diseleno lipoic acid SeL, exceeds the performance of the best 1,2-dithiolane, i.e. SA 4 as well as the best disulfide at maximal ring tension, i.e. ETP 5, with regard to both efficiency and intracellular localization. No trace of endosomal capture as with 1,2-dithiolane 4, no trace of escape into the nucleus as with ETP 5: diselenolane 7 delivers mostly to the cytosol (Fig. 9). The fluorescence images are particularly impressive with the strong quenching exerted by 1,2-diselenolanes taken into consideration (Fig. 5). The homologous 1,2-dithiolane SL 3 is clearly less performing not only than SeL 7 but also SA 4.1

A comprehensive analysis of the nature of 1,2-diselenolanes compared to 1,2-dithiolanes reveals a combination of ring-tension, selenophilicity and the high but different acidity of primary and secondary selenols as the origin of the superb performance of 1,2-diselenolane 7. Most importantly, thiol-exchange affinity chromatography is introduced to mimic and rationalize insights on thiol-mediated cellular uptake. The similar characteristics of ETP 5 and SeL 7 recorded by this method point toward an intriguing multitarget thiol hopping mechanism along so far unexplored routes to account for the efficient cytosolic delivery of these most powerful systems.22,25

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

We thank Xavier Martin-Benloch for contributions to synthesis, the Roux group for assistance with cell culture, the NMR, the MS 2.0 and the Bioimaging Platform for services, and the University of Geneva, the Swiss National Centre of Competence in Research (NCCR) Chemical Biology, the NCCR Molecular Systems Engineering and the Swiss NSF for financial support.

Notes and references