Catalyst, Not Scavenger: Reading the Organoselenium Antioxidant Literature

Rashmi Singh[1], Swarup J. Chatterjee[2]
[1] *S. N. College of Pharmacy, Babupur, Lucknow Road, Jaunpur – 222132, Uttar Pradesh, India (affiliated to Dr. A. P. J. Abdul Kalam Technical University, Lucknow)*
[2] *Professor & Principal, Department of Pharmaceutical Chemistry, S. N. College of Pharmacy, Babupur, Lucknow Road, Jaunpur – 222132, Uttar Pradesh, India.*

***Abstract*—Ebselen is the reference compound for almost every organoselenium antioxidant paper written in the last forty years, and it is routinely evaluated by assays that cannot, in principle, detect what it does. Ebselen carries no O–H and no N–H bond; it has no hydrogen atom to donate to a stable radical. What it has is a selenium atom that cycles between a selenenyl sulfide, a selenol and a selenenic acid, reducing hydroperoxide after hydroperoxide for as long as a thiol cofactor is supplied. It is a catalyst. DPPH, ABTS and FRAP contain no thiol, measure a single stoichiometric event, and report a concentration at which half of one reagent has been consumed; a catalyst has no such concentration, and the IC50 that gets tabulated is a measure of the compound's direct reducing power, not of its peroxidase-mimetic activity. This review sets out the distinction and its consequences for the design of a structure–activity study. It describes the catalytic cycle and its rate-determining step, the two assay formats that can actually see turnover — the thiophenol assay and the coupled glutathione-reductase assay — and the documented finding that the two can rank the same series in different orders, so that a paper reporting only one of them has reported a conditional result. It then examines why selenium is not a quiet bioisostere of sulfur: a selenol is more than 99 % ionised at pH 7.4 against about 11 % for a thiol of the same architecture, and replacing selenocysteine by cysteine costs native selenoenzymes more than two orders of magnitude of activity. The structure–activity literature is read against that chemistry, including the non-intuitive result that weakening the intramolecular Se···N interaction can raise catalytic activity rather than lower it. Finally, the review treats the two liabilities that any selenium series must address — indiscriminate reactivity toward protein cysteines, which has made ebselen a recurring promiscuous hit in high-throughput screens, and the narrow margin between the nutritional and toxic doses of the element itself. A reporting checklist is proposed that would make such studies comparable.**

***Index Terms*—Ebselen; organoselenium; glutathione peroxidase mimic; selenol; thiophenol assay; coupled reductase assay; DPPH; Se···N interaction; structure–activity relationship**

I. INTRODUCTION

Ebselen, 2-phenyl-1,2-benzisoselenazol-3(2H)-one, was described in 1984 as a seleno-organic compound with glutathione peroxidase-like activity [1,2]. The claim was precise and correct: in the presence of glutathione it reduced hydroperoxides catalytically, as the selenoenzyme does, rather than being consumed by them. That single property launched a field. Four decades on, ebselen remains the benchmark for new organoselenium compounds, it has completed a phase III stroke trial and reached late-stage trials in Ménière's disease, and the published analogues run into the thousands [3,4].

The field has also acquired a habit that sits awkwardly with its own founding observation. A large fraction of papers synthesising ebselen-inspired compounds evaluate them by DPPH scavenging, sometimes with ABTS and FRAP alongside, report an IC50 in µg/mL against ascorbic acid, and build a structure–activity argument on the resulting rank order. Those assays are well validated for what they measure: the capacity of a compound to hand an electron or a hydrogen atom to a coloured oxidant in a single stoichiometric encounter. Ebselen has no hydrogen atom to hand over — no hydroxyl, no amine N–H, no thiol; the selenium sits in a Se–N bond inside a five-membered ring. In an assay

containing no thiol, nothing converts it into the species that does the chemistry.

This is not an argument that such papers measure nothing. A molecule may reduce DPPH by routes other than H-atom transfer, and electron-rich substituents will register. It is an argument that what they measure is not the property their introductions claim to study, and that a rank order obtained this way carries no information about peroxidase-mimetic activity. The two quantities differ in kind, not merely in magnitude: one is a stoichiometry, the other a rate.

The confusion is easy to trace. Both properties are called antioxidant activity, both are reported as a concentration, and both appear in tables of the same shape. The careful literature keeps two terms apart — radical-trapping antioxidant capacity for the stoichiometric property, glutathione peroxidase-like activity for the catalytic one — and doing so costs nothing but a few words in the methods. This review sets out the distinction and its consequences for the design of a structure–activity study.

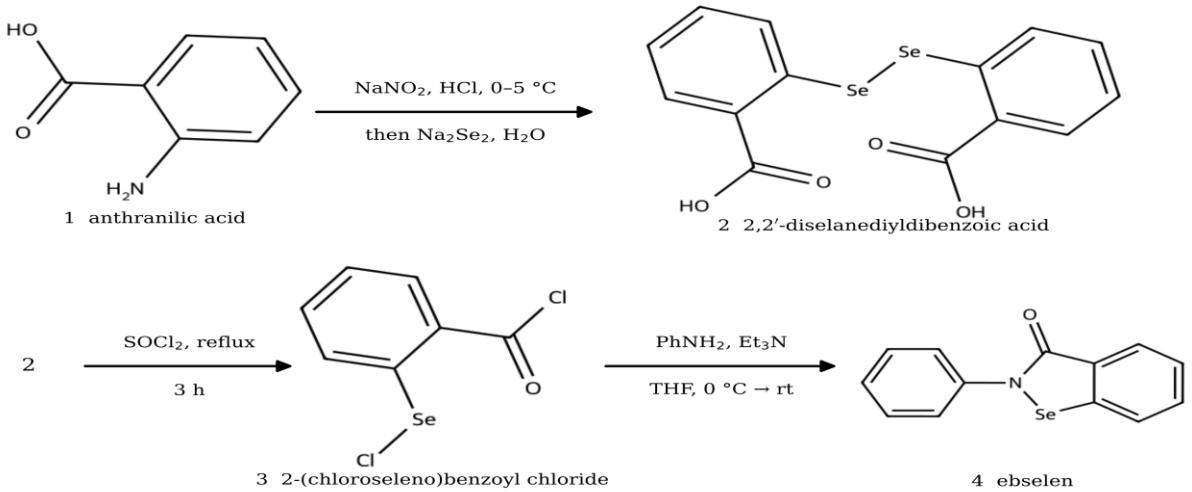
II. EBSELEN: PREPARATION AND THE CATALYTIC CYCLE

2.1 Synthesis

The standard route to benzisoselenazolones runs through anthranilic acid (Scheme 1). Diazotisation and treatment with disodium diselenide give 2,2′-diselanediyldibenzoic acid. Thionyl chloride then converts both functional groups at once, the carboxylic acids becoming acid chlorides and the Se–Se bond being cleaved to give 2-(chloroseleno)benzoyl chloride; a primary aromatic amine closes the five-membered ring in a single operation [5].

The route is short and tolerant of substitution on the aniline, which is why the ebselen literature is dominated by N-aryl variation: the last step is where diversity is cheapest. That synthetic convenience has shaped the SAR, and a series varying only the N-aryl group is exploring the part of the molecule furthest from the selenium.

Characterisation deserves a word, because selenium makes it unusually easy and the opportunity is routinely wasted. Selenium-77 is spin-1/2 with about 7.6 % natural abundance and a chemical shift range spanning thousands of parts per million, so one 77Se spectrum distinguishes a benzisoselenazolone from a diselenide, a selenol, a selenenic acid and a seleninic acid without ambiguity [36]. A study whose central claim concerns the oxidation state of selenium during turnover, but which identifies its compounds only by infrared and proton NMR, has left its most discriminating tool unused.



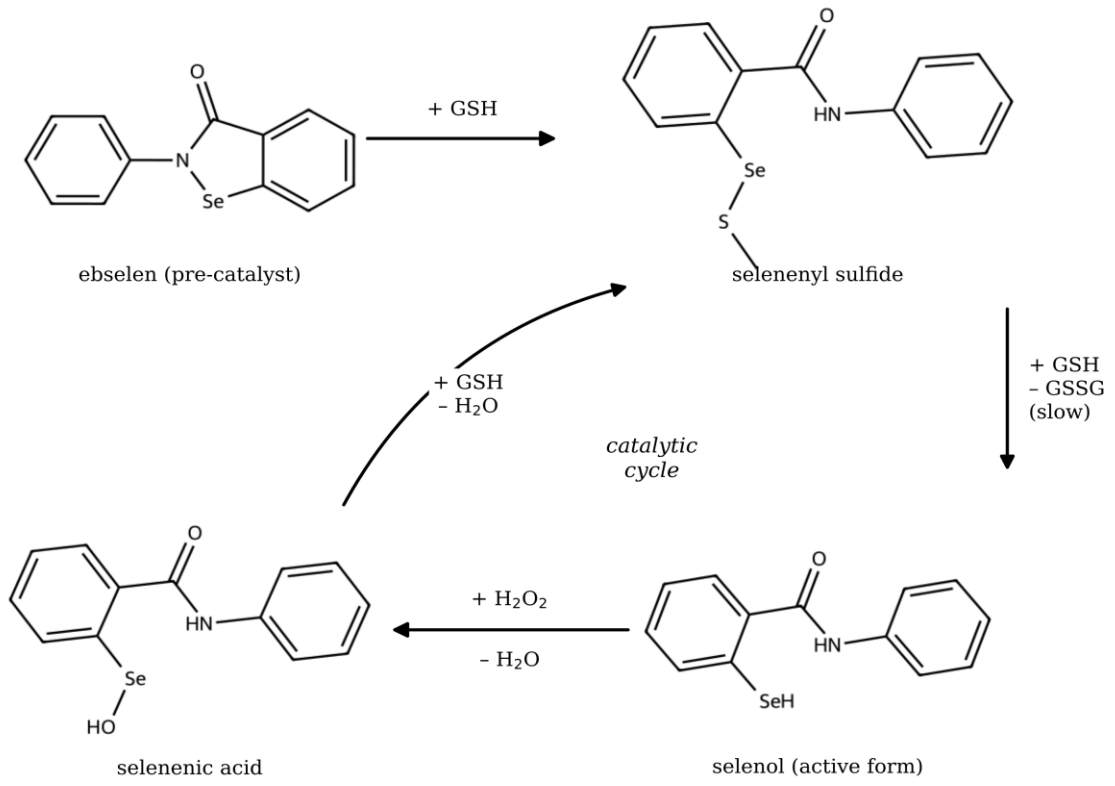
Scheme 1. Standard preparation of ebselen from anthranilic acid. Diazotisation and treatment with disodium diselenide give 2,2′-diselanediyldibenzoic acid (2); thionyl chloride converts the carboxyl groups to acid chlorides and cleaves the Se–Se bond to give 2-(chloroseleno)benzoyl chloride (3); condensation with aniline closes the benzisoselenazolone ring. Substitution is introduced at the last step, which is why N-aryl variation dominates the published series.

2.2 The catalytic cycle

Ebselen as isolated is a pre-catalyst. The cycle it enters (Scheme 2) has three steps that turn over, preceded by one that happens once [1,2,6]. The entry step is attack of a thiol on selenium, opening the Se–N bond to give a selenenyl sulfide. A second equivalent of thiol then reduces that to the selenol, releasing the disulfide; the selenol reduces the hydroperoxide and is oxidised to the selenenic acid; and thiol regenerates the selenenyl sulfide, closing the cycle [11].

The mechanistically important result, and one that explains why many mimics disappoint, is that the rate-determining step is reduction of the selenenyl sulfide to the selenol, not reaction with peroxide [11,12]. The amide carbonyl that gives ebselen its stability also coordinates to selenium through an Se···O interaction, lowering the electrophilicity at the sulfur of the selenenyl sulfide and slowing the incoming thiol. The feature that makes ebselen a well-behaved crystalline solid is the feature that throttles its turnover. Every design programme aimed at improving ebselen is negotiating that trade-off, whether or not it says so.

Three consequences follow. Ebselen's GPx activity, measured properly, is modest, and many simpler diselenides outperform it [6,7]. Because the slow step consumes thiol rather than peroxide, the measured rate depends strongly on which thiol is used and at what concentration, which makes cross-paper comparison hazardous unless conditions are matched. And ebselen is also a substrate for thioredoxin reductase, acting as a thioredoxin peroxidase mimic, which in cells may matter more than the glutathione route [41].



Scheme 2. The glutathione peroxidase-mimetic cycle. Ebselen is a pre-catalyst: the first equivalent of thiol opens the Se–N bond once, and the three steps that follow turn over. Reduction of the selenenyl sulfide to the selenol is rate-determining, because the Se···O interaction with the amide carbonyl lowers the electrophilicity at sulfur. The glutathionyl group is drawn as a methylthio group for clarity. No step in this cycle occurs in a DPPH, ABTS or FRAP assay, none of which contains a thiol.

III. SELENIUM IS NOT A QUIET BIOISOSTERE OF SULFUR

Papers in this area are often framed as replacing sulfur with selenium, implying a conservative swap. The physical chemistry says otherwise.

The decisive number is the pKa. A selenol ionises at about 5.2, a structurally matched thiol at about 8.3 [23–26]. At pH 7.4, as Fig. 1(a) shows, the selenol is 99.4 % deprotonated and the thiol 11.2 %. Since the anion performs the nucleophilic attack on peroxide, essentially all of the selenium compound is catalytically competent at physiological pH, whereas the sulfur analogue holds nine tenths of its material in an unreactive form. The selenolate is also the better nucleophile and the Se–H bond is more easily broken; these effects compound rather than cancel. Table 1 collects the comparison.

Nature's own experiment is the cleanest evidence. Replacing selenocysteine by cysteine in glutathione peroxidases, iodothyronine deiodinases and methionine sulfoxide reductase B leaves the protein folded and the site otherwise intact, and costs more than two orders of magnitude of activity [25,26]. An organism that encodes a dedicated tRNA, a specific mRNA element and a specialised elongation factor to insert one residue is paying heavily for a change that, on a bioisosterism argument, should be nearly free.

It is also worth being clear about what a mimic substitutes for. The glutathione system is a regenerating network: the peroxidase consumes reduced glutathione, glutathione reductase restores it at the expense of NADPH, and the pentose phosphate pathway supplies the NADPH [10]. A synthetic mimic inserts itself into the first step only. It brings no cofactor of its own, and in a cell with a depleted glutathione pool it will not work — which is the very condition such compounds are proposed to treat.

The practical reading is that a selenium analogue of a known sulfur compound is a new compound with new properties, not a probe of the same pharmacophore. The matched sulfur analogue is the single most informative control a selenium paper can include, and it is usually absent.

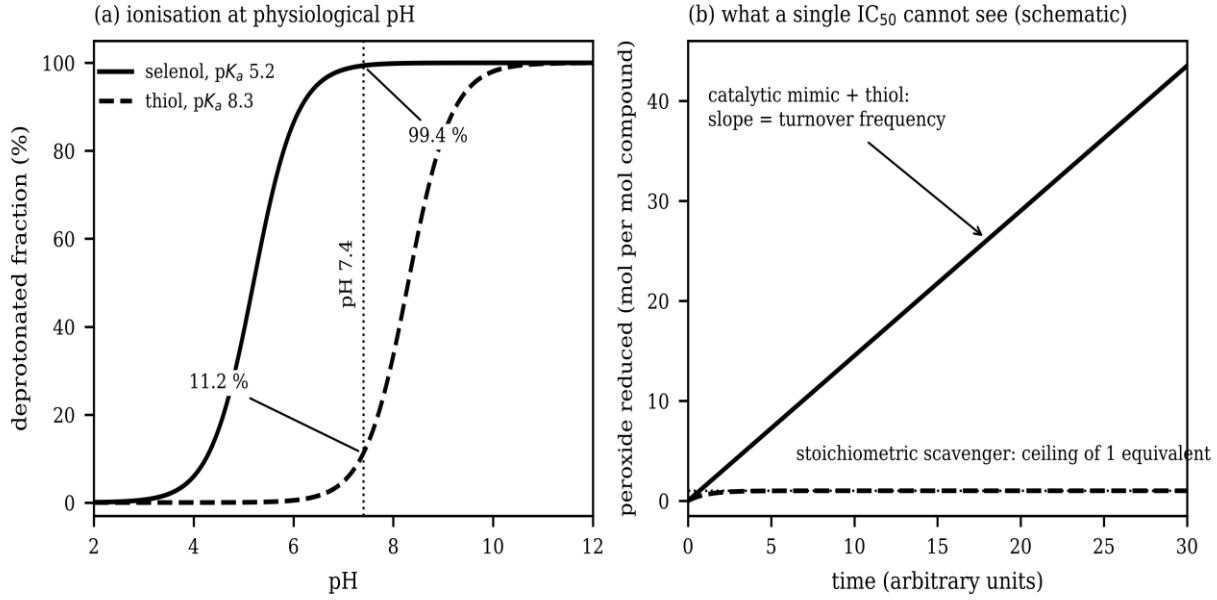


Fig. 1. Why a stoichiometric assay cannot characterise a catalytic mimic. (a) Fraction of the deprotonated, nucleophilic form as a function of pH for a selenol (pKa 5.2) and a structurally matched thiol (pKa 8.3); at pH 7.4 the selenol is 99.4 % ionised and the thiol 11.2 %. Curves computed from the Henderson–Hasselbalch relation using the pKa values in refs [23–26]. (b) Peroxide reduced per molecule of compound against time (schematic). A stoichiometric radical scavenger cannot pass one equivalent; a catalyst supplied with a thiol cofactor is limited only by the cofactor. An IC50 locates a point on the lower curve and has no counterpart on the upper one.

Table 1. Selenium against sulfur in the context of peroxidase-mimetic catalysis. The pKa values are those cited in refs [23–26]; the ionised fractions are computed from them at pH 7.4. The substitution result is from site-directed mutagenesis of glutathione peroxidases, iodothyronine deiodinases and methionine sulfoxide reductase B [25,26].

Property	Sulfur (thiol)	Selenium (selenol)	Consequence for catalysis
pKa of X–H	≈ 8.3	≈ 5.2	Selenolate is the resting state at pH 7.4; thiol is 89 % protonated
Ionised fraction at pH 7.4	11.2 %	99.4 %	Nearly all the selenium is in the nucleophilic form
Pauling electronegativity	2.58	2.55	Marginal; not the source of the difference
van der Waals radius	1.80 Å	1.90 Å	Larger, more polarisable, softer nucleophile
X–H bond strength	Higher	Lower	Selenol is more easily oxidised and more easily re-formed
Oxidised form	Sulfenic acid, reversible	Selenenic acid, reversible but over-oxidises more readily	Dead-end seleninic acid at high peroxide
Sec→Cys substitution in native selenoenzymes	—	> 100-fold loss of activity	The element is not interchangeable in the active site

IV. WHAT THE ANTIOXIDANT ASSAYS ACTUALLY MEASURE

4.1 The stoichiometric assays

DPPH is a stable nitrogen-centred radical, deep violet, that turns pale yellow on reduction [27]. ABTS is used as its radical cation and decolorises on reduction [28]. FRAP measures reduction of a ferric tripyridyltriazine complex at low pH [29]. All three are electron- or hydrogen-transfer assays run as a single oxidant–antioxidant pairing, and all three report a concentration at which a fixed proportion of oxidant has been consumed [30,31].

Three features matter here. The assays contain no thiol; they are stoichiometric, so the answer is an amount rather than a rate; and their mechanism is solvent-dependent, DPPH switching between hydrogen-atom transfer and sequential proton-loss electron transfer with the hydrogen-bonding ability of the medium, so the same compound gives different values in methanol and ethanol [32].

Against a peroxidase mimic the first two are disqualifying. With no thiol, ebselen is never converted to the selenol and the cycle never starts; and even if it did, the assay could not express the result. Fig. 1(b) makes the point: a stoichiometric scavenger cannot exceed one equivalent of oxidant per molecule, whereas a catalyst with a thiol cofactor is limited only by the cofactor. A single-timepoint absorbance at fixed concentration cannot distinguish a slow catalyst from a fast one, and cannot see catalysis at all.

A secondary problem compounds the first. Results are usually given as IC50 in µg/mL against ascorbic acid. Mass concentration is the wrong unit for mechanistic comparison: ebselen's molar mass is 274.2 g/mol against 176.1 for ascorbic acid, so a µg/mL comparison understates the selenium compound by about 1.6-fold before any chemistry is considered. Ascorbic acid is also a poor comparator for a compound proposed to act catalytically, being the archetypal stoichiometric reductant; ebselen itself is the conventional standard for a peroxidase assay [6,7].

None of this makes a DPPH experiment worthless. It makes it a measurement of direct radical-trapping capacity — a real and separately interesting property — which should be labelled as such and reported alongside, not instead of, a peroxidase assay.

4.2 The thiophenol assay

The chemical assay of Iwaoka and Tomoda uses thiophenol as the stoichiometric reductant and hydrogen peroxide as oxidant in an organic solvent, following the appearance of diphenyl disulfide

spectrophotometrically [14]. The initial rate relative to the uncatalysed background is the measure of activity, and much of the comparative data in the field comes from it; the camphor-derived selenenamide of Back and Dyck is one example [33].
Its virtues are simplicity, no enzymes, and a quantity that is a rate. Its limitation is that thiophenol is far more acidic than glutathione, so a larger fraction is present as thiolate, and the medium is non-aqueous. A compound whose slow step is thiol attack on a protected selenium will therefore look better here than it would with a biological thiol at pH 7.4.

4.3 The coupled reductase assay
The assay developed in the original ebselen work and formalised subsequently is enzymatic [2,15]. Glutathione, glutathione reductase and NADPH are combined with the test compound and a hydroperoxide. The mimic reduces the peroxide at the expense of glutathione, the reductase regenerates the glutathione at the expense of NADPH, and NADPH disappearance is followed at 340 nm. Because the glutathione pool is continuously restored, many turnovers can be observed and the slope is a genuine catalytic rate under near-physiological conditions.
The limitations mirror those above. The assay is sensitive to anything inhibiting glutathione reductase, which selenium compounds may well do; without a no-peroxide control it will score a compound that merely oxidises glutathione directly; and the enzyme requirement makes it more variable between laboratories.

4.4 Why the two correct assays disagree
One might expect two assays measuring turnover to agree on rank order. They do not always: some ebselen derivatives show no detectable activity in the thiophenol assay yet match or exceed ebselen in the coupled reductase assay, and the reverse is also reported [7,34,38].
The explanation follows from Section 2.2. The two formats load different steps. A highly reactive thiol in organic solvent tends to make peroxide reduction rate-limiting; glutathione in water at pH 7.4 tends to make thiol exchange rate-limiting. A structural change that accelerates one step and retards the other moves a compound in opposite directions in the two assays. The disagreement is not an artefact to be averaged away; it is information about which step a substituent acts on.
The operational consequence is that GPx-like activity is a property of a compound in an assay, not of a compound. Table 2 summarises what each format requires and what it can see. A paper reporting a single assay has reported a conditional result and should say so.

Table 2. What each assay format requires and what it reports. Only the last two contain a thiol, and only the last two return a rate. The first three measure direct radical-trapping or reducing capacity, which is a real property but a different one; results from them should be labelled accordingly and not used to rank peroxidase-mimetic activity [27–32].

Assay	Oxidant	Thiol present	Quantity returned	Detects catalytic turnover?
DPPH	DPPH radical	No	IC50 (stoichiometric)	No
ABTS	ABTS radical cation	No	Trolox equivalents	No
FRAP	Fe(III)–TPTZ, pH 3.6	No	Fe(II) equivalents	No
Thiophenol assay	H2O2 in organic solvent	Yes (PhSH)	Initial rate of PhSSPh formation	Yes
Coupled reductase assay	H2O2 or t-BuOOH, pH 7.4	Yes (GSH, regenerated)	Rate of NADPH loss at 340 nm	Yes

V. STRUCTURE–ACTIVITY RELATIONSHIPS, READ AGAINST THE MECHANISM

5.1 The Se·N interaction and its non-monotonic effect
The interaction between selenium and a neighbouring nitrogen or oxygen is the most-discussed structural variable in the field [5,36], and the usual narrative is that stronger is better. The evidence does not support the simple form of that claim. In the comparative study of Mugesh and du Mont, mimics with weaker Se···N interactions — second-order perturbation energies below about 7 kcal/mol — showed relatively

higher catalytic activities than those with strong interactions [6].

The reason is the one already identified: a strong donor interaction stabilises the intermediates and in particular reduces the electrophilicity of the selenenyl sulfide, the species that must react in the slow step. The interaction is a tuning parameter with an optimum, not a quantity to maximise. This is the clearest example in the field of a plausible design heuristic falsified by rate measurements on a matched series — which is what an SAR study is for, and what a DPPH rank order cannot do.

5.2 Cyclic benzisoselenazolones against open diselenides

The second axis is whether the selenium sits in a ring. Diaryl diselenides are simpler to make, generally more active than ebselen, and correspondingly less stable and more promiscuous [7,13]. Introducing an ortho amine, as in the amine-based diselenides of Fig. 2, installs the Se···N interaction in an open framework and lets it be tuned by substitution at nitrogen [14,36]. Ebselen sits at the stable end of this spectrum, and its diselenide — formed in situ by disproportionation of the selenenyl sulfide — is itself catalytically competent [11,12], so the species turning over during an assay may be a mixture, and a trace that accelerates after a lag is a sign of it rather than of experimental error.

Allyl and cyclic selenides form a third class. Back and Moussa showed that allyl selenides owe their activity to cyclic seleninate esters generated in situ by oxidation [13] — a different catalytic species altogether, and a reminder that the compound weighed into the cuvette is not necessarily the compound doing the work. Water-soluble cyclic selenides were developed partly to avoid the diselenide intermediate, and show that an Se–Se bond is not required for turnover [35].

A sober assessment of the class is available and should be read by anyone entering it. Orian and Toppo surveyed four decades of organochalcogen peroxidase mimetics and called the field a long story of a promise unfulfilled: thousands of compounds, many with in vitro activities exceeding ebselen's, and no approved drug [37]. Activity measured in a cuvette with an artificial thiol has repeatedly failed to translate, and one reason is that the optimised quantity was chosen for convenience rather than for its relation to the biology.

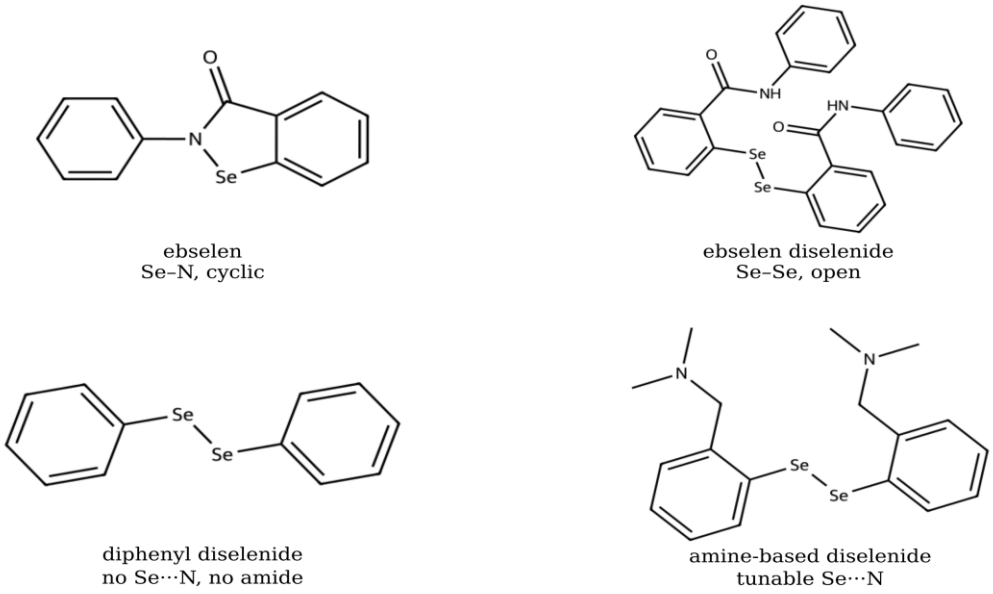


Fig. 2. Representative organoselenium antioxidant scaffolds. Ebselen holds selenium in a five-membered ring closed by an Se–N bond; its diselenide is the open, dimeric form generated in situ during turnover and is itself catalytically competent. Diphenyl diselenide is the reference open diselenide and has neither an amide nor an Se···N contact. Bis[2-(dimethylaminomethyl)phenyl] diselenide carries an ortho amine whose donation to selenium can be tuned by substitution, the structural variable discussed in Section 5.1.

5.3 Substituent effects and what a matched series requires

N-aryl variation is the best-populated part of the literature for the synthetic reason given in Section 2.1. The reported effects are real but modest, consistent with a substituent remote from the reaction centre acting largely through the amide [34]; electron-withdrawing groups on the benzisoselenazolone ring itself, closer to selenium, have larger effects.

For a dissertation-scale project the design question is not how many compounds to make but how to vary one thing at a time. Table 3 sets out the structural classes and the question each is positioned to answer. Twelve N-aryl variants answer one narrow question well; four compounds spanning cyclic, open, strong-Se···N and weak-Se···N, each with its matched sulfur analogue, answer a mechanistic question and are a better use of the same bench time.

Table 3. Structural classes in the organoselenium antioxidant literature and the specific question each is positioned to answer. A small series spanning several rows is more informative than a large series confined to one, and the last row is the control that is almost always omitted.

Structural class	Defining feature	Question the class is positioned to answer
Benzisoselenazolones (ebselen type)	Se–N in a five-membered ring, amide Se···O contact	How much does ring closure cost in turnover, and how much stability does it buy?
N-aryl-substituted ebselen analogues	Variation remote from selenium	Can the amide be used to tune the slow step indirectly?
Diaryl diselenides	Open Se–Se, no amide	What is the activity of the scaffold without the stabilising interaction?
Amine-based diselenides	Tunable ortho Se···N donation	Where is the optimum of the non-monotonic Se···N relationship?
Cyclic and water-soluble selenides	Se in a saturated ring, no Se–Se	Can the selenenic acid be handled without a diselenide intermediate?
Matched sulfur analogues of any of the above	S in place of Se	Is the activity attributable to selenium at all?

VI. THE UNPRODUCTIVE FATES OF THE CATALYST

Two side reactions determine how long a mimic keeps working, and both appear in Scheme 3. The first is disproportionation: two molecules of the selenenyl sulfide give the diselenide and the disulfide [11,12]. For ebselen this is not a loss, since the diselenide is active, but the catalyst's identity changes during the assay.

The second is over-oxidation, and it is a genuine dead end. If hydroperoxide is in excess over thiol, the selenenic acid is oxidised further to the seleninic acid, which is not reduced back under assay conditions. This is the chemical basis of a common and avoidable failure: an assay run with too much peroxide, or one in which the thiol has been exhausted, shows a rate decaying to zero that is then reported as low activity. The diagnostic is to vary thiol concentration at fixed peroxide — a genuine low activity is insensitive to it, whereas over-oxidation recovers.

Order of addition matters for the same reason. Because the pre-catalyst must first be opened by thiol, a cuvette charged with compound and peroxide before the thiol is added will show a lag or nothing at all. Pre-incubating compound with thiol and starting with peroxide is the correct protocol, and its omission from published methods is one reason nominally identical assays give different numbers in different laboratories. A third process matters in cells rather than cuvettes: at high peroxide and low thiol the selenium can be stripped from the molecule altogether, releasing inorganic species whose toxicology is quite different from the parent's [8,9].

VII. THIOL REACTIVITY, PROMISCUITY AND SELECTIVITY DATA

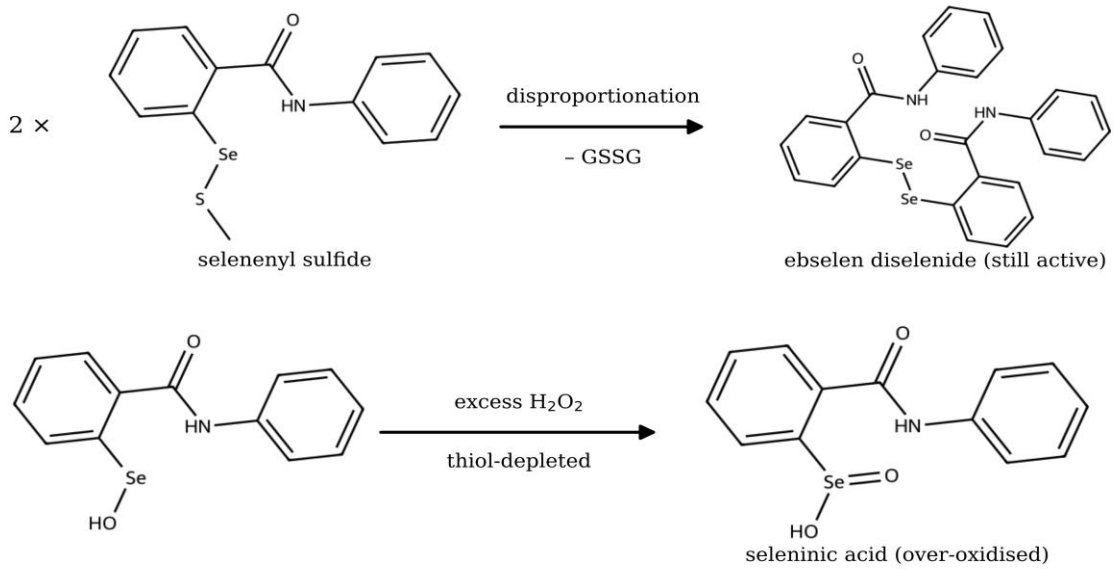
The property that makes ebselen a catalyst — rapid, reversible reaction with thiols — does not distinguish

glutathione from the cysteine residue of a protein. Ebselen forms selenenyl sulfide adducts with accessible protein cysteines readily, and this is the mechanism behind a large share of the activities attributed to it.

The clearest demonstration came from early screening against the SARS-CoV-2 main protease, where ebselen was identified as a potent hit [16] and was subsequently shown, with several other cysteine-reactive compounds, to be a nonspecific promiscuous inhibitor whose apparent potency collapsed under appropriate counter-screens [17]. The lesson predates the pandemic: a compound that covalently and reversibly modifies cysteine will score in a high proportion of target-based assays, and that is not evidence of target engagement [39,40].

The counter-screens are standard and cheap. A reducing agent such as dithiothreitol in the buffer abolishes apparent inhibition by a cysteine-reactive compound; jump dilution distinguishes reversible from irreversible binding; detergent rules out aggregation; and a time-dependent curve with a steep Hill slope is itself a warning. For an antioxidant study the implications are that cell-based readouts of protection may reflect modification of signalling proteins rather than peroxide removal, and that observed cytotoxicity may arise from thiol depletion rather than selenium toxicity — distinguishable by co-incubation with N-acetylcysteine.

The constructive version of the point is that reversibility is a design variable. The Se–N and Se–S bonds involved are labile, and changes that raise the barrier to adduct formation with bulky protein thiols while leaving the small-molecule cofactor reaction fast are a legitimate and largely unexplored selectivity strategy.



Scheme 3. Competing fates. Top: disproportionation of two molecules of the selenenyl sulfide gives ebselen diselenide and the disulfide; for ebselen the diselenide is still an active catalyst, so the species turning over during an assay may be a mixture. Bottom: when peroxide is in excess over thiol the selenenic acid is oxidised further to the seleninic acid, which is not reduced back under assay conditions. This is the usual cause of a catalytic rate that decays to zero and is then misreported as low activity.

VIII. SELENIUM TOXICOLOGY AND THE THERAPEUTIC WINDOW

Selenium is an essential trace element with an unusually narrow margin between requirement and harm. The recommended dietary allowance for adults is 55 µg per day and the tolerable upper intake level 400 µg per day, a ratio of about seven [21]; the upper level was re-evaluated by the European Food Safety Authority in 2023 [22]. Chronic excess produces selenosis, with hair and nail loss, garlic breath and peripheral neuropathy.

That margin is set by inorganic and simple organic species and does not transfer directly to a stable heterocycle such as ebselen, which is well tolerated at doses far above the nutritional range. But it means three things for a synthetic programme. Any compound liable to release selenide or selenite in vivo inherits the element's toxicology. Cytotoxicity data are not optional here as they might be for a purely phenolic antioxidant, and a selectivity index against a normal cell line should accompany every activity figure. And the diselenides, generally the more active mimics, are generally also the more cytotoxic [8,9], so activity and tolerability move together in a way the activity table alone conceals.

A formulation point belongs here too. Ebselen is very poorly water-soluble, and the flat, rigid, donor-free heterocycle that gives it stability is what keeps it out of solution. Reported activities therefore depend on a dimethyl sulfoxide stock, and the final solvent concentration is a variable that should be stated and matched across compounds; a compound that precipitates at the working concentration returns a flattering IC50 and an uninterpretable rate.

The clinical record corrects both over-enthusiasm and over-caution. Ebselen completed a placebo-controlled phase III trial in acute ischaemic stroke in Japan [18], entered trials as a lithium mimetic in bipolar disorder on the strength of its inhibition of inositol monophosphatase [19], and has been evaluated in noise-induced hearing loss [20] and, as SPI-1005, through phase III in Ménière's disease, with more than nine hundred patients dosed across that programme. What it has not done in forty years is reach approval — and its in vitro GPx activity, properly measured, was never the reason it advanced.

IX. WHAT A DEFENSIBLE STUDY SHOULD REPORT

The preceding sections converge on a short list, written for a project of dissertation scale in which the argument must be carried by the design rather than by volume.

Report the assay that matches the claim. If the claim is peroxidase-mimetic activity, run the thiophenol assay or the coupled reductase assay and state the thiol, its concentration, the peroxide, the solvent and the pH. If DPPH or ABTS is also run, label it as radical-trapping capacity and do not derive the structure–activity argument from it.

Run both GPx formats where possible and report any discordance rather than suppressing it; a compound active in one and not the other is telling you which step the substituent affects. Include the matched sulfur analogue — the only control that tests the bioisosterism premise directly, and a cheap one. If the sulfur compound comes within a factor of two, the selenium is not where the activity lies, and that is worth reporting.

Include a no-catalyst background rate, because the uncatalysed thiol–peroxide reaction is not negligible, and a no-peroxide control, which separates peroxide reduction from direct thiol oxidation. Vary the thiol concentration at a minimum of two levels to distinguish genuinely low activity from over-oxidation. Characterise the compounds by 77Se NMR and report the shifts.

Report cytotoxicity on a normal cell line beside every activity figure and compute a selectivity index; for a selenium series this is not a formality, since the more active mimics are often the more toxic and an activity table without a toxicity column flatters the worst candidates. Give concentrations in molar units, state the solvent and its final concentration, and confirm that nothing precipitated at the top of the range.

If docking is used, validate the protocol by redocking a co-crystallised ligand and reporting the RMSD, and do not offer a docking score as evidence of antioxidant activity: the catalytic chemistry described here is not a binding event and no scoring function models it. Finally, state the number of independent replicates and the dispersion, not the standard error of three technical repeats — rate measurements in thiol-containing media are sensitive to adventitious metal ions and dissolved oxygen, and between-day variation usually exceeds within-day variation.

X. CONCLUSION

The organoselenium antioxidant literature contains an unusually clean example of a measurement problem. Ebselen does something specific and well understood: with a thiol cofactor it cycles through a selenol and reduces hydroperoxides catalytically, limited by the rate at which thiol attacks its selenenyl sulfide. None of the three assays most commonly used to characterise its analogues contains a thiol,

and none reports a rate. The mismatch is not subtle and is not controversial among those who work on the mechanism; it persists because the stoichiometric assays are cheap, fast and familiar.

The remedy is not to abandon them but to stop asking them a question they cannot answer. A study that measures turnover in a thiol-containing system, varies one structural feature at a time against a matched sulfur control, reports both GPx formats when they disagree, and places a cytotoxicity figure beside every activity figure will produce a smaller table than the conventional alternative and a far more defensible one.

The non-monotonic behaviour of the Se···N interaction is the standing demonstration of what such work yields. The intuitive rule was that a stronger intramolecular donor interaction should give a better mimic; rate measurements on matched compounds showed the opposite above a threshold, identified the slow step, and explained why the most stable compound in a series is not the best catalyst. That is a result only the right assay can produce, and it is available to any project willing to measure a rate instead of an endpoint.

REFERENCES

[1] Müller A, Cadenas E, Graf P, Sies H. A novel biologically active seleno-organic compound—I. Glutathione peroxidase-like activity in vitro and antioxidant capacity of PZ 51 (ebselen). Biochem Pharmacol. 1984;33(20):3235–3239.

[2] Wendel A, Fausel M, Safayhi H, Tiegs G, Otter R. A novel biologically active seleno-organic compound—II. Activity of PZ 51 in relation to glutathione peroxidase. Biochem Pharmacol. 1984;33(20):3241–3245.

[3] Sies H. Ebselen, a selenoorganic compound as glutathione peroxidase mimic. Free Radic Biol Med. 1993;14(3):313–323.

[4] Parnham MJ, Sies H. The early research and development of ebselen. Biochem Pharmacol. 2013;86(9):1248–1253.

[5] Mugesh G, du Mont W-W, Sies H. Chemistry of biologically important synthetic organoselenium compounds. Chem Rev. 2001;101(7):2125–2179.

[6] Mugesh G, du Mont W-W. Structure–activity correlation between natural glutathione peroxidase (GPx) and mimics: a biomimetic concept for the design and synthesis of more efficient GPx mimics. Chem Eur J. 2001;7(6):1365–1370.

[7] Bhabak KP, Mugesh G. Functional mimics of glutathione peroxidase: bioinspired synthetic antioxidants. Acc Chem Res. 2010;43(11):1408–1419.

[8] Nogueira CW, Zeni G, Rocha JBT. Organoselenium and organotellurium compounds: toxicology and pharmacology. Chem Rev. 2004;104(12):6255–6285.

[9] Nogueira CW, Rocha JBT. Toxicology and pharmacology of selenium: emphasis on synthetic organoselenium compounds. Arch Toxicol. 2011;85(11):1313–1359.

[10] Arteel GE, Sies H. The biochemistry of selenium and the glutathione system. Environ Toxicol Pharmacol. 2001;10(4):153–158.

[11] Sarma BK, Mugesh G. Antioxidant activity of the anti-inflammatory compound ebselen: a reversible cyclization pathway via selenenic and seleninic acid intermediates. Chem Eur J. 2008;14(34):10603–10614.

[12] Sarma BK, Mugesh G. Glutathione peroxidase (GPx)-like antioxidant activity of the organoselenium drug ebselen: unexpected complications with thiol exchange reactions. J Am Chem Soc. 2005;127(32):11477–11485.

[13] Back TG, Moussa Z. Diselenides and allyl selenides as glutathione peroxidase mimetics. Remarkable activity of cyclic seleninate esters produced in situ by the oxidation of allyl ω-hydroxyalkyl selenides. J Am Chem Soc. 2003;125(44):13455–13460.

[14] Iwaoka M, Tomoda S. A model study on the effect of an amino group on the antioxidant activity of glutathione peroxidase. J Am Chem Soc. 1994;116(6):2557–2561.

[15] Wilson SR, Zucker PA, Huang R-RC, Spector A. Development of synthetic compounds with glutathione peroxidase activity. J Am Chem Soc. 1989;111(15):5936–5939.

[16] Jin Z, Du X, Xu Y, et al. Structure of Mpro from SARS-CoV-2 and discovery of its inhibitors. Nature. 2020;582:289–293.

[17] Ma C, Hu Y, Townsend JA, et al. Ebselen, disulfiram, carmofur, PX-12, tideglusib, and shikonin are nonspecific promiscuous SARS-

CoV-2 main protease inhibitors. ACS Pharmacol Transl Sci. 2020;3(6):1265–1277.

[18] Yamaguchi T, Sano K, Takakura K, et al. Ebselen in acute ischemic stroke: a placebo-controlled, double-blind clinical trial. Stroke. 1998;29(1):12–17.

[19] Singh N, Halliday AC, Thomas JM, et al. A safe lithium mimetic for bipolar disorder. Nat Commun. 2013;4:1332.

[20] Kil J, Lobarinas E, Spankovich C, et al. Safety and efficacy of ebselen for the prevention of noise-induced hearing loss: a randomised, double-blind, placebo-controlled, phase 2 trial. Lancet. 2017;390(10098):969–979.

[21] Institute of Medicine. Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids. Washington, DC: National Academies Press; 2000. Chapter 7, Selenium.

[22] EFSA Panel on Nutrition, Novel Foods and Food Allergens. Scientific opinion on the tolerable upper intake level for selenium. EFSA J. 2023;21(1):e07704.

[23] Huber RE, Criddle RS. Comparison of the chemical properties of selenocysteine and selenocystine with their sulfur analogs. Arch Biochem Biophys. 1967;122(1):164–173.

[24] Byun BJ, Kang YK. Conformational preferences and pKa value of selenocysteine residue. Biopolymers. 2011;95(5):345–353.

[25] Arnér ESJ. Selenoproteins—what unique properties can arise with selenocysteine in place of cysteine? Exp Cell Res. 2010;316(8):1296–1303.

[26] Reich HJ, Hondal RJ. Why nature chose selenium. ACS Chem Biol. 2016;11(4):821–841.

[27] Blois MS. Antioxidant determinations by the use of a stable free radical. Nature. 1958;181:1199–1200.

[28] Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med. 1999;26(9–10):1231–1237.

[29] Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. Anal Biochem. 1996;239(1):70–76.

[30] Apak R, Özyürek M, Güçlü K, Çapanoğlu E. Antioxidant activity/capacity measurement. 1. Classification, physicochemical principles, mechanisms, and electron transfer based assays. J Agric Food Chem. 2016;64(5):997–1027.

[31] Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. J Agric Food Chem. 2005;53(6):1841–1856.

[32] Foti MC, Daquino C, Geraci C. Electron-transfer reaction of cinnamic acids and their methyl esters with the DPPH radical in alcoholic solutions. J Org Chem. 2004;69(7):2309–2314.

[33] Back TG, Dyck BP. A novel camphor-derived selenenamide that acts as a glutathione peroxidase mimetic. J Am Chem Soc. 1997;119(9):2079–2083.

[34] Bhabak KP, Mugesh G. Synthesis, characterization, and antioxidant activity of some ebselen analogues. Chem Eur J. 2007;13(16):4594–4601.

[35] Kumakura F, Mishra B, Priyadarsini KI, Iwaoka M. A water-soluble cyclic selenide with enhanced glutathione peroxidase-like catalytic activities. Eur J Org Chem. 2010;(3):440–445.

[36] Mukherjee AJ, Zade SS, Singh HB, Sunoj RB. Organoselenium chemistry: role of intramolecular interactions. Chem Rev. 2010;110(7):4357–4416.

[37] Orian L, Toppo S. Organochalcogen peroxidase mimetics as potential drugs: a long story of a promise unfulfilled. Free Radic Biol Med. 2014;66:65–74.

[38] Bhowmick D, Mugesh G. Insights into the catalytic mechanism of synthetic glutathione peroxidase mimetics. Org Biomol Chem. 2015;13(41):10262–10272.

[39] Baell JB, Holloway GA. New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. J Med Chem. 2010;53(7):2719–2740.

[40] Dahlin JL, Nissink JWM, Strasser JM, et al. PAINS in the assay: chemical mechanisms of assay interference and promiscuous enzymatic inhibition observed during a sulfhydryl-scavenging HTS. J Med Chem. 2015;58(5):2091–2113.

[41] Zhao R, Masayasu H, Holmgren A. Ebselen: a substrate for human thioredoxin reductase strongly stimulating its thioredoxin-dependent peroxidase activity and a superfast thioredoxin

oxidant. Proc Natl Acad Sci USA. 2002;99(13):8579–8584.