Targeting thioredoxin reductase with organometallics

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**TARGETING THIOREDOXIN REDUCTASE WITH
ORGANOMETALLICS**

KOH WEI XIANG

SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

2021

TARGETING THIOREDOXIN REDUCTASE WITH ORGANOMETALLICS

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B.Sc. (Hons), 2016

SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Doctor of Philosophy

2021

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Authorship Attribution Statement

This thesis contains material from one paper published in the following peer-reviewed journal(s) / from papers accepted at conferences in which I am listed as an author.

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The contributions of the co-authors are as follows:

- Prof Leong and Prof Holmgren provided the initial project direction.
- Prof Leong edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Dr Coppo.
- I co-designed the study with Dr Coppo and Ms Kong and performed all the laboratory work at the School of Physical and Mathematical Sciences and Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm. I also analyzed the data.
- All experiments were done jointly by me and Ms Kong.

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Abstract

The thioredoxin (Trx) system is a key antioxidant system to the regulation of oxidative stress in cells. Thioredoxin reductase (TrxR) is an enzyme which is central to the Trx system and is upregulated in cancer cells, making it a potential target in anti-cancer therapy. Its active site contains an S-Se bond (Cys⁴⁹⁷-Sec⁴⁹⁸). Since both sulphur and selenium are soft bases, we hypothesise that they may be targeted by organometallic compounds. In this work, two aspects on targeting TrxR with organometallics have been examined: (a) Developing organometallic complexes and derivatives of known drugs that act on TrxR, and (b) mechanistic and kinetic studies on the reaction of organometallic complexes with the S-Se bond.

For the first aspect, two groups of organometallic complexes have been studied. The first group are the metallocenyl derivatives of ebselen, a known substrate of TrxR. They are found to inhibit TrxR with an IC₅₀ of $2.5 \pm 0.3 \mu\text{M}$ in a pure protein assay; they have a higher affinity towards TrxR ($K_m = 0.4 \mu\text{M}$) than the natural substrate, Trx. The second is the carbonyl cluster Os₃(CO)₁₀(NCMe)₂ which has already been shown to induce apoptosis. It is found to interact with selenyl-sulphide and selenides, besides its known reactivity with disulphides and sulfhydryl groups. It inhibits TrxR with an IC₅₀ of $5.3 \pm 0.9 \text{ mM}$ as a non-substrate inhibitor, is selective even in the presence of high concentrations of GSH, and it has also been shown to inhibit TrxR in cell lysate. These results suggest that the inhibition of TrxR is a potential apoptotic pathway.

The second aspect concerns the hypothesis that heterodinuclear organometallic complexes may react selectively with the hetero-dichalcogenide S-Se bond over the homo-dichalcogenide bonds S-S and Se-Se. Mechanistic and kinetic studies on the reactivity of homo/hetero bimetallic Ru and/or Mo complexes (M-M', where M/M'=Ru/Mo), with two small-molecule mimics for the dichalcogenides, viz., PhEEPh and 1,8-naphthalenedichalcogenides, have been

carried out. The reactions are initiated through photocleavage, by visible light, of the metal-metal bonds of the bimetallic complexes. While the Mo-Mo compound reacts faster with Se-containing molecules, the Ru-Ru compound preferred S-containing molecules; the Ru-Mo compound exhibits comparable kinetics for all three E-E' molecules. A similar trend is observed for the naphthalene derivatives although the rates are significantly slower, postulated to be due to a chelate effect.

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Compound Labelling Scheme

Number	Compound
1	<p>M = Fe (a), Ru (b), Os (c)</p>
2
3	<p>E = Se (a), S (b)</p>
4	<p>M = Fe (a), Ru (b), Os (c)</p>
5
6	<p>n = 0 (a), 1 (b)</p>
7
MM'	<p>M, M' = Ru (n = 2) or Mo (n = 3)</p>

ME^R		M, M' = Ru (n = 2) or Mo (n = 3) E = S or Se R = H, Me, OMe

	Se⁻	SH	COOH
EE'		E, E' = S or Se	
E^RE'R'		E, E' = S or Se R, R' = H, Me, OMe	
napEE'		E = S or Se	

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Chapter 1. The Thioredoxin System and Bio-organometallic Chemistry

Anti-cancer therapy remains a priority in pharmaceutical research.¹ There are many approaches in treating and retarding the spread of cancerous cells, one of which is to inhibit enzymes that are responsible for the proliferation of cells.²⁻⁵ It is widely accepted that both oxidative stress and hypoxia are common features of cancer cells.⁶ It is thus not surprising to find high levels of antioxidant proteins, such as thioredoxin (Trx), in tumors. Studies have shown elevated expression of Trx in several primary cancers, including lung, cervix, pancreatic and breast cancer.⁶ The up-regulation of Trx has been suggested to benefit the proliferation and malignant progression of tumors, acting as one of the enhancers of cancer cell growth.⁶

1.1 The Thioredoxin System

Thioredoxin (Trx) is a class of small redox proteins that are known to be present in most, if not all, organisms. Trx plays an important role in many biological processes such as redox signalling and is important in cell proliferation cycles. Trx acts as an antioxidant by acting as the facilitator for the reduction of other proteins through cysteine thiol-disulfide exchange. It is a 12-kD oxidoreductase enzyme which contains a dithiol-disulfide active site responsible for most of its biological activities. Trx is oxidised to its disulfide form while performing its biological activities. The thereby oxidatively deactivated Trx is reduced by the flavoenzyme thioredoxin reductase (TrxR) back to its dithiol form in a NADPH-dependent reaction. Nicotinamide adenine dinucleotide phosphate, or commonly known as NADP^+ , is a cofactor required in many anabolic reactions. An example is the reaction with TrxR where NADPH (the reduced form of NADP^+) acts as the reducing agent in a cascade of biological reactions through which Trx is ultimately kept in its reduced form.

The thioredoxin system has been described as ubiquitous and exists in all living cells.⁷ The physiological functions of Trx varies from common fundamental reactions to different specialized functions, depending on the organism. Recent findings have demonstrated the role of Trx in defence against oxidative stress and in the control of apoptosis.^{8, 9} Most of these biological functions depends on the disulphide reductase activity of Trx and thereby the availability of NADPH and the activity of TrxR. The differing roles of Trx in diverse organisms have been described in great detail; that of its oxidoreductase activity is shown below (Figure 1.1).¹⁰

Figure 1.1. Oxidoreductase activities of the thioredoxin system.

Thioredoxin reductases are one of the few enzymes that reduce thioredoxin. TrxR can be subdivided into two classes; one occurs in bacterial and some eukaryotes, and the other in animals. Human TrxR is known to catalyse the NADPH-dependent reduction of thioredoxin disulphide and numerous other oxidized cell constituents. With its low substrate specificity, TrxR also contributes to redox homeostasis and is involved in the prevention, intervention and repair of damage by H₂O₂-based oxidative stress. The carcinoma cellular environment is frequently under oxidative or hypoxic stress. These stresses occur because of the abnormal vascular systems which result in unstable oxygen delivery, leading to complex oxygenating cycle. This environment upregulates Trx expression to support regulation of the abnormal oxidative environment.^{6, 11} The inhibition of

TrxR activity has negative effects on the proliferation and malignant progression of tumors, making it a viable drug target.¹²⁻¹⁴

1.2 Active Site of Thioredoxin Reductase

One appealing characteristic of mammalian TrxR, as opposed to other enzymes that are responsible for cancer cell proliferation, is that it contains a Sec amino acid residue. In contrast, bacterial TrxR does not contain Sec amino acid residue. This Sec residue, which contains a selenol moiety, is unique to TrxR and hence drugs can be designed to specifically target the selenol functional group so as to increase its selectivity.^{10, 14, 15} TrxR contains two active sites - Cys⁴⁹⁷-Sec⁴⁹⁸ and Cys⁵⁹-Cys⁶⁴ (Figure 1.2). The former is responsible for reducing Trx while the latter acts as a reductant for the former. Zhong *et al.* has demonstrated that TrxR contains the crucial - Gly-Cys⁴⁹⁷-Sec⁴⁹⁸-Gly C-terminal sequence which has been confirmed by mass spectroscopy.¹⁶ A mutant version of TrxR in which Sec⁴⁹⁸ was replaced by a Cys displayed a 100-fold lower k_{cat} for Trx reduction.¹⁶ TrxR is known to exist in both its reduced and oxidised forms; in the former, the S-Se bond is reduced to thiol (SH) and selenol (SeH) by its cofactor, NADPH. Under physiological conditions, the selenol is deprotonated into selenide (Se(-)).

Figure 1.2. Schematic up view of the human TrxR active site.

The currently accepted mechanism for the TrxR system is depicted below (Figure 1.3). The inactive form **A** of TrxR is first reduced to its active form **B** by NADPH. This in turn reduces Trx and is transformed to intermediate **C**. Upon release of the reduced Trx, the TrxR is oxidised into

D. Intra-enzymatic reduction takes place at this stage whereby Cys⁵⁹ reduces the selenium-sulphur bond across Cys⁴⁹⁷-Sec⁴⁹⁸ to form **E**. The cycle is then closed when **E** is reduced to **B** by NADPH.

Flavin adenine dinucleotide (FAD) acts as a redox cofactor throughout this cycle.

Figure 1.3. Postulated mechanism of Thioredoxin Reductase.

1.3 Current Approaches to Targeting Thioredoxin Reductase

The design of small drug molecules to target TrxR, namely the Cys⁴⁹⁷-Sec⁴⁹⁸ residue, has been difficult due to the small chemical difference between an S-S bond and an S-Se bond. Most, if not all, of the drugs targeting TrxR as their mode of action involve exploiting the high nucleophilicity of the selenide (Se(-)) in the reduced form of TrxR to form a covalent bond between Sec⁴⁹⁸ and the drug.^{9, 17, 18, 19, 20} Two representative classes of drugs targeting TrxR are organic substrates such as ebselen and metal-based complexes.

1.3.1 Organic Substrates - Ebselen

N-substituted benzoselenazol-3(2H)-ones have been shown to exhibit a broad spectrum of biological activities, including anti-inflammatory and antioxidant activity, and are also believed to be anticancer agents.^{1-5, 15, 21-27} One particular compound which has attracted considerable interest is 2-phenyl-1,2-benzisoselenazol-3(2H)-one, commonly known as ebselen, a substrate of TrxR. The sulphur analogue, ebsulfur, is also known and has been shown to inhibit growth of bacteria such as *S. aureus* (Figure 1.4).

Figure 1.4. Structures of ebselen and ebsulfur.

Ebselen was originally developed as a treatment for stroke, but was abandoned in the final phase of clinical trials due to its poor efficacy.²⁸ This suggests that although it is not efficient as a treatment for stroke, it is clinically safe. Structure activity relationship (SAR) studies have shown

that the selenium atom in ebselen is essential for its activity.²³ Organic derivatization of ebselen and ebsulfur have also shown that the potency decreases with any substitution of the two phenyl rings. These suggest that we have to retain the selenium or sulphur atom and the phenyl rings. Ebselen competes well against Trx as a substrate for TrxR, showing comparable K_m value, and is reduced to ebselen-TrxR diselenide by NADPH (Figure 1.5).¹⁵ While in the case of bacterial TrxR, ebselen acts as an inhibitor.²¹

Figure 1.5. Inhibition of TrxR by ebselen.

1.3.2 Metal-based Drugs – Arsenic Trioxide and Auranofin

Metal based drugs such as Arsenic Trioxide (ATO) and auranofin (AF) are shown to inhibit TrxR. Arsenic trioxide is an effective cancer therapeutic drug for acute promyelocytic leukemia and is shown to have potential anticancer properties against an extensive array of solid tumours. Studies have shown that ATO inhibits TrxR irreversibly, inducing oxidative stress resulting in cell apoptosis.^{29, 30} Similarly, the gold-based drug AF inhibits TrxR, inducing oxidative stress which results in cell apoptosis.³¹ The mechanism of action is proposed to be the inhibition of TrxR via the Sec residue.^{14, 30, 32-36}

1.4 Chemistry of the Dichalcogen Bonds

As discussed above, the active site of TrxR contains a sulphur-selenium (S-Se) bond in its oxidised form, and exists as a thiol (SH) and selenide (Se(-)) in its reduced form. This bond is unique to mammalian TrxR as no other enzymes or proteins are known to contain this particular bond.^{10, 13, 16, 37} Most of the drugs known to target TrxR are based on the selenide and thiol moiety being highly nucleophilic, as exemplified by ebselen which forms a covalent bond with the selenide moiety of Sec, or auranofin which interacts with the thiol moiety of the cysteine residues.

A mode of action that involves reaction with selenide results in low selectivity since any other enzyme or protein containing exposed Sec (like TrxR) would likely interact. Similarly, the close homologue of S-Se moiety, the disulphide (S-S) bond is ubiquitous in proteins. Thus the reaction of a drug with S-S, SH and/or Se(-) is probably responsible for the side-effects in most chemotherapies.^{13-16, 29, 34, 38} This suggests the selective targeting of S-Se bond to be highly desirable as it would equate to selectively targeting the active site of hTrxR. However, it has been difficult to distinguish between an S-S bond and an S-Se bond due to the small chemical difference between sulphur and selenium.³⁹ Sulphur and selenium can act as both electrophiles or nucleophiles in chemical reactions, depending on the reactants and reaction environment. Studies have shown that selenium is a better electrophile, nucleophile and leaving group in typical organic chemical reactions, thus making selenocysteine favourable as an active site for oxidoreductase-like catalysis.^{39, 40} To date, however, the chemistry of the selenyl-sulphide (SeS) bond has not been vastly studied although it has been identified as one of the key studies required to understand the intracellular targets and how they impact signalling.⁴⁰ Compounds containing SeS bonds are generally used as precursors to other chalcogen-containing molecules.⁴¹

A thorough study of the kinetic and mechanistic differences between these bonds will allow us a better understanding of the chemistry and hence to design better drugs to target not only the S-Se bond, but their reduced forms as well. One possible method to address this issue is to employ organometallic compounds. These can be fitted with various types of ligands to fine-tune the chemistry to fit that of the hTrxR active site. Based on Pearson's hard-soft acid-base (HSAB) concept, the softer selenium (in comparison with sulphur) would tend to bind to a softer organometallic metal centre. A heterobimetallic species can be designed such that two different transition metals with similar chemical difference to that of the sulphur-selenium bond which may favour the sulphur-selenium bond over a disulphide bond.

1.5 Bioorganometallic Chemistry

Organometallic chemistry lies in-between organic chemistry and inorganic chemistry. It involves the study of compounds containing metal-carbon bond(s). Studies of organometallic compounds were initially driven by their significance in catalysis and industrial applications. These compounds are generally perceived as air and moisture sensitive, their potential relevance in biological application was largely disregarded. The term bioorganometallic chemistry, first coined in 1985, refers to the study of organometallics in biological application and it has attracted much attention since then. Their biological application includes, but not limited to, pharmaceuticals, bio-catalysis, bio-imaging and sensing.⁴²⁻⁴⁴

In pharmaceutical applications, compounds with transition metal moiety have been introduced as drugs for various treatment including cancer. One good example is cisplatin, which has been used as treatment for several cancers. One typical strategy in employing transition metals is to introduce an organometallic fragment onto a known pharmacologically-active organic compound, thereby modifying its property. A good example is the ferrocenyl moiety, which has

similar chemistry to that of the phenyl ring but with an additional property not available with the phenyl ring - its redox activity.⁴⁵⁻⁴⁷ Two examples that illustrate this are ferrocifen and ferroquine (Figure 1.6).^{45, 48}

Figure 1.6. Examples of ferrocenyl derivatives of tamoxifen and chloroquine.

Tamoxifen is an FDA-approved anti-cancer drug that is used in the treatment of estrogen-receptor-positive (ER+) breast carcinoma. Two out of three breast cancers are tested positive for ER+, while the remaining are estrogen-receptor-negative (ER-). Ferrocifen has been found to be biologically active towards both ER+ and ER- breast carcinomas.^{46, 48} More recently, the osmium derivative of ferrocifen, *viz.*, osmocifen, has also been shown to inhibit TrxR via the Sec residue.⁴⁹ Chloroquine is an antimalarial drug that is now facing drug resistance. Ferroquine presents itself as the next generation drug for the treatment of malaria.⁴⁵

A more recent example of a potential new class of organometallic anti-cancer drugs are those based on osmium carbonyl clusters, such as, $\text{Os}_3(\text{CO})_{10}(\text{NCMe})_2$. This has been shown to exhibit high cytotoxicity for both ER+ and ER- breast cancer cells in the micromolar range.⁵⁰⁻⁵³ The mode of action remains unclear, although it has been shown that $\text{Os}_3(\text{CO})_{10}(\text{NCMe})_2$ interacts with intracellular -SH and -COOH residues and with various protected amino acids in the following reactivity order: thiol > carboxylic acid > amide. This suggests the potential of $\text{Os}_3(\text{CO})_{10}(\text{NCMe})_2$ interacting and inhibiting the cysteine or selenocysteine in TrxR as one of the plausible apoptotic pathway.

1.6 Aim and Objective

From the discussion above, inhibition of thioredoxin reductase is an important strategy to selectively target cancer cells in cancer therapy. This has been done thus far by targeting the Sec residue of TrxR but suffers from poor selectivity as various other enzymes and proteins contain Sec residues. The oxidized form of TrxR exhibits a SeS bond that is unique to TrxR, making it an ideal drug target, however the chemistry of this bond is not well studied. Organometallic chemistry may provide an answer to selectively target the SeS bond as the metal and/or ligands can be fine-tuned to match its chemistry. Indeed, one known cytotoxic organometallic species that may potentially inhibit TrxR as its apoptotic pathway is $\text{Os}_3(\text{CO})_{10}(\text{NCMe})_2$. At the same time, the modification of known drug candidates with organometallic moieties has been shown to be an effective strategy to the discovery of new classes of drugs. Modification of the known substrate, ebselen, with a metallocenyl moiety as an inhibitor of TrxR is thus attractive.

The aim of this project is to examine the potential of organometallic compounds in the inhibition of TrxR with the following objectives:

- 1) To apply the strategy of derivatizing a known TrxR substrate, namely, ebselen, with a metallocenyl moiety
- 2) To investigate TrxR inhibition by triosmium carbonyl clusters
- 3) To examine the relative reactivity of the dichalcogenides (SeS, SS and SeSe) with homo- and heterodinuclear organometallic complexes

1.7 References

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Chapter 2. Metallocenyl Derivatives of Ebselen

2.1 Ferrocenyl derivatives of pharmacologically active compounds

Driven by the success of cisplatin as a treatment for a number of cancers, there has been great interest in the development of compounds with a transition metal moiety as drugs.¹ As mentioned in chapter 1, a common strategy is to replace a phenyl ring with the ferrocenyl moiety, as exemplified by ferrocifen and ferroquine (Figure 2.1).²⁻⁴ In both cases, their enhanced effectiveness has been ascribed to the redox property of the ferrocenyl group with the introduction of redox active metal centre, iron. Since TrxR acts through a redox mechanism, it is likely that such a modification can also be applied effectively to a known substrate of TrxR.

Figure 2.1. Examples of ferrocenyl derivatives of tamoxifen and chloroquine.

Ebselen is a known substrate of TrxR and competes well with its natural substrate Trx. The selenium-nitrogen bond in ebselen is essential for its activity, and the phenyl ring attached to the nitrogen can be replaced with a ferrocene (Figure 2.2). The sulphur derivate of ebselen, ebsulfur, has also been shown to exhibit antibacterial properties via inhibition of bacterial TrxR.

Figure 2.2. Ebselen, ebsulfur and their ferrocenyl derivatives, **1a** and **2**.

Since the activity of ebselen and ebsulfur centres around the N-X bond (where X = Se or S, respectively), it is desirable to have the ferrocenyl moiety to be close to this bond. The heavier homologues of the group VIII metals, *viz.*, Ru and Os, will also be of interest as they are expected to exhibit similar chemistry but with different redox potentials. The difference in the redox potentials will likely influence the efficacy of the drug molecule, while the syntheses should be similar to those of the ferrocenyl derivatives.

2.2 Synthesis

Many straightforward approaches to the synthesis of ebselen are known (Scheme 2.1).⁵⁻⁹ The metallocenyl moiety can be introduced by replacing the appropriate reagent with the metallocenyl counterpart; a similar synthetic route can also be applied for the ebsulfur analogues.

Scheme 2.1. Synthesis routes to ebselen.

The metallocenyl derivatives of ebselen and ebsulfur that were investigated are summarized below, together with the retrosynthetic analysis; the first disconnection is that of the X-N and C(O)-N bonds (Scheme 2.2).

Scheme 2.2. Retrosynthetic analysis of target molecules.

The five precursors for the four target molecules are summarized above (Scheme 2.2). All are either not commercially available, or are too expensive, but may be prepared from commercially available chemicals via multistep routes.¹⁰⁻¹⁶

2.2.1 Preparation of the chalcogenyl chlorides 3

The reported synthetic route to precursor **3a** is shown below (Scheme 2.3).¹³⁻¹⁵ Base hydrolysis of phthalimide afforded a α -aminoacid intermediate. An *in situ* Hofmann rearrangement then afforded anthranilic acid. The identity of the product was confirmed by comparison of its ¹H NMR spectroscopic data with the published values.¹⁵ Conversion of anthranilic to the diazonium salt, followed by nucleophilic substitution with basic aq. Na₂Se₂, afforded the 2,2'-diselenobisbenzoic acid. Completion of the synthesis of **3a** was accomplished by treatment of the 2,2'-diselenobisbenzoic acid in thionyl chloride, with DMF as catalyst; the seleno and acyl chlorides were both installed in this single step.¹⁴

Scheme 2.3. Synthetic route to compound **3a**.

The precursor **3b** is reported to be prepared easily from commercially available 2,2'-dithiobisbenzoic acid; the 2,2'-dithiobisbenzoic acid was first converted to the acyl chloride by treatment with thionyl chloride, followed by treatment with sulfonyl chloride to cleave the disulphide bond (Scheme 2.4).¹¹

Scheme 2.4. Synthetic route to compound **3b**.

2.2.2 Preparation of the aminometallocenes **4**

The aminometallocenes of the group VIII elements (**4**) can be prepared from the reported synthetic route shown in Scheme 2.5.^{12, 16}

where M = Fe, Ru or Os.

Scheme 2.5. Synthetic route to the aminometallocenes **4**.

Attempts at replacing the more reactive *t*-butyllithium with *n*-butyllithium resulted in either no derivatization of the metallocene, or a mixture of mono-, di- and tri-substituted derivatives. *t*-Butyllithium afforded the mono-substituted metallocene in a relatively high yield of ~50%, together with unreacted metallocene.^{12, 16} No purification of this mixture was necessary at this stage as the unreacted metallocenes will not participate in the next reaction; the amount of iodo metallocene present can be calculated from the ¹H NMR spectra. The amination was carried out via the metallocenyl halide in a one-pot reaction, with aqueous NH₃ as the nitrogen source and CuI/Fe₂O₃ as co-catalysts.¹² The identities of the products were confirmed by ¹H NMR spectroscopy and the spectra obtained coincides with the literature values.¹²

This procedure has been applied to iodoosmocene and yielded the as-yet unreported aminoosmocene (**4c**). The identity compound **4c** is confirmed by its ¹H NMR spectroscopy; the nine cyclopentadienyl proton resonances are present in a 2:2:5 ratio in the 4 – 6 ppm region, with a broad peak at 2.50 ppm for the NH₂ protons. The spectral characteristics are similar to its Fe and Ru homologues.

2.2.3 The Coupling Reaction

The coupling of **3a** with aniline to form ebselen was first attempted to establish the optimal reaction conditions.^{5, 6, 8, 9, 17} It was found that simple mixing in DCM was sufficient; the progress

of the reaction was monitored by TLC, and the identity of ebselen was confirmed by its ^1H NMR and mass spectral data with literature values.⁵⁻⁷ Similar reaction conditions were employed for the coupling of **3a** and **4a** to afford **1a** (Scheme 2.6); the sulphur analogue **2** was similarly prepared from **3b**. While the ruthenium analogue **1b** and osmium analogue **1c** have been successfully synthesized using the same reaction conditions and identified on the basis of the ^1H NMR and EI-MS data, it was observed that **1c** was unstable, decomposing upon exposure to air.

Scheme 2.6. Coupling of **3a** and **4a** to give **1a**.

The structures of both **1a** and **2** have been determined crystallographically; the ORTEP diagram depicting the molecular structure of **1a** is shown in Figure 2.3, together with selected bond parameters for both. The Se-N and S-N bond lengths are similar to those of the parent compounds ebselen and ebsulfur^{18, 19}, thus demonstrating that there is no significant structural effects from the ferrocenyl moiety. The Se-N and S-N bond lengths of **1a** and **2**, respectively, have a difference of about 0.18 Å suggesting **1a** to be more reactive than **2**. This difference is also observed in their parent molecules.

Bond Length/Angles ($\text{\AA}/^\circ$)	1a / (ebselen)	2 / (ebsulfur)
E-N1 (E = Se (1a), S(2))	1.901(7) (1.896(3))	1.722(2) (1.718(2))
N1-C11	1.346 (12) (1.359 (5))	1.374 (2) (1.380 (2))
C11-C12	1.479 (12) (1.464 (6))	1.465 (3) (1.468 (3))
C12-C13	1.377 (13) (1.413(5))	1.382 (3) (1.394 (3))
C13-E (E = Se (1a), S(2))	1.892 (9) (1.892 (4))	1.740 (2) (1.739 (2))
C11-O1	1.234 (11) (1.237 (4))	1.226 (2) (1.222 (2))
N1-C10	1.399 (11) (1.444 (5))	1.374 (2) (1.430 (2))

Fe1-Cp (Centroid)	1.644	1.637
C13-E-N1 (E = Se (1a), S(2))	85.0 (3) (85.8 (2))	90.26 (8) (90.5 (1))
E-N1-C11 (E = Se (1a), S(2))	115.3 (5) (116.0 (2))	115.7 (1) (116.1 (1))
N1-C11-C12	112.1 (8) (110.8 (3))	108.6 (2) (108.3 (2))
C11-C12-C13	114.8 (8) (116.6 (3))	113.4 (2) (113.4 (2))
C12-C13-E (E = Se (1a), S(2))	112.8 (6) (110.7 (3))	111.9 (2) (111.8 (1))

Figure 2.3. ORTEP plots showing the molecular structure of **1a**. Thermal ellipsoids are drawn at 50% probability. Hydrogen atoms have been omitted for clarity.

The structures of **1a-c** and ebselen were also studied computationally by density functional theory (DFT) at the M06/def2TZVP level of theory; selected bond parameters are tabulated below (Figure 2.4). The bond parameters for **1a-c** are comparable except for the Fe-Cp (centroid) distance, which is 0.18 Å shorter in **1a** than that in **1b** and **1c**. They thus suggest that the identity of the metal has little structural effects on the ebselen moiety.

Figure 2.4. DFT Calculated bond parameters of ebselen and **1a-c**. For **1a-c**, the bond parameters are listed in descending order, from **1a-c**.

2.3 Biochemical Studies

The air-sensitivity of **1c** precluded biochemical studies on it, thus all the biochemical studies were carried out with **1a** and **1b**, collectively referred to as **1** henceforth.

2.3.1 Cytotoxicity

The anti-proliferative activities of **1a** and **1b** on ER+ breast carcinoma (MCF-7) were determined using the MTS assay; they were found to have IC₅₀'s of 75 μ M and 49 μ M, respectively, which were lower than that of ebselen (217 μ M) (Table 2). This suggests that introduction of the metallocenyl moiety increases the cytotoxicity of the parent molecule.

Table 2.1. Cell Proliferation assay of **1**, ebselen and Os-bis-ACN.

	MCF-7 (μ M)
Compound 1a	75 $\pm$ 9
Compound 1b	49 $\pm$ 5
Ebselen	216.9 $\pm$ 1.4
Os ₃ (CO) ₁₀ (NCMe) ₂	8.10 $\pm$ 0.80

2.3.2 TrxR assays

The enhanced cytotoxicity prompted further studies into the activity of the compounds towards TrxR. The two main properties of the compounds that are of interest are:

1. Do the compounds inhibit TrxR?
2. Are the compounds substrate of TrxR?

TrxR Inhibition

DTNB reacts with sulfhydryl groups to form 2 TNB²⁻ which has an absorption band at 412 nm.^{20, 21} It is also a substrate of TrxR; DTNB is reduced by TrxR to form TNB²⁻ with NADPH as the reductant source (scheme 2.7).

Scheme 2.7. Redox cycle of TrxR with DTNB as substrate.

The DTNB end-point assay is used to check if the test compounds are inhibitors of TrxR. In the assay, the compound of interest is first incubated with TrxR in the presence of NADPH before DTNB is added. The rate of formation of TNB is then measured periodically. A slower rate of formation as compared with a negative control and/or vehicle suggests inhibition of the enzyme; the assay for **1b** is shown in Figure 2.5. It was found that both **1a** and **1b** are inhibitors of TrxR, with IC₅₀ of 4.2 ± 0.3 and 2.5 ± 0.3 μM, respectively.

Figure 2.5. DTNB reduction assay of **1b**. TrxR were incubated with different concentrations of the compound **1b** in presence of 400uM of NADPH (4 mg/mL). After 15 minutes, 2.2 mM of DTNB was added and the absorbance of the samples was recorded at 412nm every 10s for 5 min.

TrxR Substrate Study

A substrate of TrxR will consume NADPH when it is introduced into a TrxR system. We can test if a compound of interest is a substrate of TrxR by the NADPH consumption assay.^{20, 21} This involves incubating the compound with TrxR and NADPH. A decrease in the concentration of NADPH would suggest that it is a substrate. The concentration of NADPH can be monitored through the absorption at 340 nm, the absorption maxima of NADPH.^{20, 21} It was found that both **1a** and **1b** resulted in faster consumption of NADPH compared with the positive control, ebselen. This suggests that the compounds are substrates of TrxR. The K_m values of the compounds were measured using Lineweaver-Burk plots to be 0.6 and 0.4 μM , respectively (Appendix A9-10).

These values are lower than that of ebselen and Trx (2.5 and 2.0 μM , respectively).^{21, 22} The Lineweaver-Burk plot is a plot of $1/[S]$ vs $1/v$ where $[S]$ is the concentration of substrate (**1**) and v is the reaction velocity. The reaction velocity is measured on a UV-vis spectrometer with the DTNB assay as described earlier.^{20, 21} The lower K_m values of the metallocenyl derivatives suggest that the derivatization of ebselen with the metallocenes increases its affinity for TrxR, and hence these derivatives are potential competitive inhibitors.

Figure 2.6. NADPH consumption assay for **1b**. Measurements of NADPH consumption were made at 340 nm (A_{340}), with TE buffer, TrxR (10 nM), BSA (0.1 mg/mL), and NADPH (40 $\mu\text{g/mL}$). The compound is substrate of TrxR as their A_{340} decreased rapidly, compared to the rapid decrease for ebselen, a known substrate.

The inhibition and substrate studies suggest the metallocenyl derivatives of ebselen to inhibit TrxR as a competitive inhibitor. Although the kinetics of the inhibition were not measured,

we can derive from the data that TrxR is inhibited in both concentration and time dependence manner. The inhibition is likely to be, similar to ebselen, reversible as the compound acts as a substrate.

2.3.3 Selectivity Studies

There are several potential selectivity issues with drugs targeting selenocysteine residues, one of which is that selenocysteine has little chemical difference from cysteine. It is therefore of interest to examine if **1** is selective towards Sec-containing TrxR over other Cys-containing enzymes and proteins such as glutathione reductase (GR) and glutathione (GSH), which are either structurally similar to TrxR or are present in relatively high abundance *in vivo*.

Glutathione reductase (GR) is homologous to TrxR; the main difference between them is that the active site of GR is across Cys⁵⁸ and Cys⁶³ and lacks the C-terminal extension containing Cys-Sec as found in TrxR.^{23, 24} GR is an enzyme that catalyses the reduction of glutathione disulphide (GSSG) to its reduced form glutathione (GSH); its biological function is similar to TrxR, to act as a reductase. The interaction of **1** with GR can be examined by measuring the NADPH consumption in the glutaredoxin system; GR consumes NADPH as the source of reductant in its catalytic cycle. (scheme 2.8).

Scheme 2.8. NADPH consumption by GR, measured at A₃₄₀.

The activity of GR can be measured by monitoring the consumption of NADPH through the absorption maximum at 340 nm for the latter; an inhibited enzyme will show lower consumption of NADPH. The results show that NADPH was consumed by GR rapidly (similar to control), indicating that **1** did not interact with GR (Figure 2.7) and hence they are selective for TrxR over GR.

Figure 2.7. NADPH consumption of **1b** in GR assay. Measurements of NADPH consumption were made at 340 nm (A₃₄₀), with TE buffer, GR (10 nM), BSA (0.1 mg/mL), and NADPH (40 μg/mL).

Glutathione (GSH) is a cysteine-containing tripeptide within the glutaredoxin system that is present at a rather high concentration (1-10 mM cf 0.16 pM of TrxR) in the cell environment.²⁵ ²⁶ It is therefore possible that it will impede the interaction of **1** with TrxR. To study this possibility, the compounds were incubated with GSH for a period of time before measurement of the amount

of free GSH using the DTNB assay. As DTNB can interact with the sulfhydryl group in GSH, the amount of TNB²⁻ formed (as measured at 412 nm) is indicative of the amount of free GSH. It was found that minor interaction with GSH was observed only at high concentrations of **1** (Figure 2.8). This suggests the possibility of GSH interaction with high concentrations of **1**.

Figure 2.8. Measurement of DTNB reduction by GSH after incubating **1b** with GSH (100 μM) in TE buffer, followed by the addition of DTNB (1.8 mM) in EtOH.

2.4 Conclusion

Metallocenyl analogues of ebselen (**1a-c**) and ebsulfur (**2**) were synthesized successfully. Compounds **1a** and **1b** were found to be more cytotoxic than the parent ebselen towards the MCF-7 cell lines. They were also found act as both substrates and inhibitors of TrxR with remarkable low K_m values, suggesting that they have higher affinity for TrxR than the natural substrate Trx, and were selective over cysteine-containing GR and GSH.

2.5 Experimental

2.5.1 General Experimental

All reactions and manipulations were carried out under an argon atmosphere using standard Schlenk techniques unless otherwise stated. Solvents that were used for reactions were distilled over the appropriate drying agents under argon before usage unless otherwise stated. A ^1H NMR spectra were recorded on a Joel ECA 400 or ECA 400SL at 400 MHz and referenced to the residual proton resonance of CDCl_3 , unless otherwise stated. ESI MS spectra were recorded on a Thermos Finnigan LCQ Fleet Mass Spectrometer. Preparative TLC separations were carried out on Merck silica gel 60 Å F 254 20 x 20 cm^2 plates. $\text{Os}_3(\text{CO})_{10}(\text{ACN})_2$ and ebselen was prepared according to literature methods.^{27, 28} All other reagents were used as received from commercial suppliers. Mammalian TrxR with approximately 14 U/mg was from IMCO Ltd. TE buffer (50 mM Tris-HCl pH 7.5, 2mM EDTA) was prepared by diluting 1 mM Tris-HCl and 0.2 M EDTA in deionized (DI) water. Stock solutions were prepared as the following: 62.5 mM DTNB in EtOH; BSA (10 mg/mL) in DI water; TrxR (1 mM), NADPH (40 mg/mL), GSH (100 mM), GSSG (10 mM) and Trx (20 μM) respectively in TE buffer. Each bioassay was analyzed in triplicates and the values were obtained from at least two separate experiments with the mean and standard error calculated. The IC_{50} for TrxR inhibition was determined from a sigmoidal dose response (variable slope) equation using the GraphPad Prism 7 software.

2.5.2 Cell Culture

Experimental cultures of breast (MCF-7) were obtained from the American Type Culture Collection (ATCC) and cultured in tissue culture dishes (Corning, NY) at 37°C in 5% CO_2

atmosphere. Phosphate-buffered saline (PBS) was obtained from Gibco. The breast cancer cells MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Biowest L0106, France) supplemented with 10% fetal bovine serum (FBS; Gibco, NY), 1% L-glutamine (PAA Laboratories, Austria), and 1% penicillin/streptomycin (HyClone, UT).

2.5.3 Synthesis of anthranilic acid

This reaction is not required to be carried out under inert atmosphere. Bromine (3.4 ml, 132 mmol) was added to a solution of sodium hydroxide (48 ml, 6M) in an Erlenmeyer flask cooled in an ice bath. Phthalimide (10 g, 4.4 mmol) was added and the mixture was then allowed to warm up to room temperature, and then heated at 80 °C for 20 min. It was then filtered, the filtrate cooled in an ice bath, and the pH adjusted to 3-4 with conc HCl. The precipitate obtained was collected by filtration as white crystals. Yield: 4.8 g (80%).

^1H NMR δ 7.93 (d, J = 8.2, 1H, ArH), 7.31 (t, J = 6.9, 1H, ArH), 6.70 – 6.66 (m, 2H, ArH).

2.5.4 Preparation of basic Na₂Se₂ solution

A solution of sodium borohydride (60 mg, 1.57 mmol) and sodium hydroxide (100 mg, 2.50 mmol) in water (12 mL) cooled in ice bath was added to a suspension of selenium (1 g, 12.6 mmol) and sodium hydroxide (0.5 g, 14.67 mmol) and stirred at room temperature for 1 h. The mixture was then slowly heated to 110 °C and maintained at this temperature for 30 min before it was cooled to room temperature slowly. The dark red solution was ready for use without further treatment.

2.5.5 Synthesis of 2,2'-diselenobisbenzoic acid

Sodium nitrite (1 g, 14.5 mmol) in water (12 mL) was added dropwise to a solution of anthranilic acid (1.8 g, 13.1 mmol) in 37% HCl (2 mL) and water (14 mL), cooled in ice bath with vigorous stirring. The mixture of resulting diazonium salt was stirred for 20 min before it was added dropwise to the Na₂Se₂ solution prepared in step 3. The mixture was stirred for 30 min at room temperature, then slowly heated to 100 °C and held at this temperature for 5 min before cooling down to room temperature slowly. The mixture was then filtered over Celite, the filtrate was acidified with 10% HCl acid to a pH of 4-5 and the precipitate form was collected by filtration. The precipitate was suspended in methanol and refluxed for 20 min. The suspension was filtered, the filtrate was evaporated in vacuo yielding the desired compound as brown crystals. Yield: 1.7 g (65%).

¹H NMR (DMSO-*d*₆) δ 8.00 (d, *J* = 7.8, 2H, ArH), 7.65 (d, *J* = 8.2, 2H, ArH), 7.42 (t, *J* = 8.2, 2H, ArH), 7.31 (t, *J* = 7.8, 2H, ArH). ESI-MS, *m/z*: 401 [M]⁺

2.5.6 Synthesis of o-chloroseleno benzoyl chloride, 3a

This reaction is not required to be carried out under inert atmosphere. 2,2'-diselenobisbenzoic acid (1 g, 2.5 mmol) was refluxed for 2 h in thionyl chloride (10 mL) with DMF (0.5 mL). Thionyl chloride was removed in vacuo and the resulting oily residue was suspended in hexane. The suspension was filtered and the filtrate was evaporated in vacuo to afford **3a** as yellow crystals, m.p. 64 – 66 °C. Yield: 900 mg (72%).

¹H NMR δ 8.35 (d, *J* = 8.2, 1H, ArH), 8.10 (d, *J* = 8.2, 1H, ArH), 7.80 (t, *J* = 8.2, 1H, ArH), 7.50 (t, *J* = 8.2, 1H, ArH).

A similar procedure with 2,2'-dithiobisbenzoic acid (1.5 g, 4.90 mmol), DMF (20 μ L), 1.0M solution of sulfuryl chloride (5 mL, 5 mmol) and thionyl chloride (2.5 mL) dissolved in 1,2-dichloroethane (10 mL) gave, upon solvent removal, **3b** as yellow solids. Yield = 1.25 g (63%).

^1H NMR δ 8.33 (m, 1H, ArH), 7.92 (m, 1H, ArH), 7.76 (m, 1H, ArH), 7.42 (m, 1H, ArH).

2.5.7 Synthesis of metallocenes

The metal trichloride and absolute ethanol (8 mL) were placed in a schlenk tube equipped with a magnetic stirring bar. Freshly cracked cyclopentadiene (5 mL, 60 mmol) was added, followed by zinc powder (2.6 g, 40 mmol) whereupon the reaction mixture turned dark grey. This was stirred at room temperature for 2 h, filtered in air through celite, washing through with toluene (15 mL). The filtrate was evaporated to dryness, the residue redissolved in toluene, filtered through silica gel and the clear filtrate evaporated to dryness to afford the product.

With ruthenium trichloride (1.00 g, 4.00 mmol), ruthenocene was obtained as a white solid.

Yield: 600 mg (64%). ^1H NMR δ 4.55 (s, 10 H, R_cH)

With osmium trichloride (1.00 g, 3.40 mmol), osmocene was obtained as a white solid. Yield: 326 mg (30%). ^1H NMR δ 4.72 (s, 10 H, O_cH)

2.5.8 Synthesis of iodometallocenes

A sample of the metallocene and KO^tBu (20 mg, 0.24 mmol) were charged into an oven-dried 100 mL carius tube previously purged with argon. Dry THF (20 mL) was added and the resulting solution was cooled to -78°C in a dry ice/acetone cold bath with stirring. A solution of ^tBuLi in pentane (1.7M, 2 mL, 3.4 mmol) was added dropwise. The solution turns a bright yellow, indicating the formation of lithiometallocene, and was allowed to stir for an additional 30 min at the same temperature. Solid I₂ (400 mg, 1.8 mmol) was then added and the reaction mixture was left to reach room temperature, stirred for an additional 24 h, and then concentrated in vacuo. The crude product was purified by column chromatography on silica using hexane as eluent.

Table 2.2. Experimental and characterisation data for the iodometallocenes.

Metallocene (mg, mmol)	Iodometallocene (mg, %)	¹ H NMR (ppm)	Colour
Ruthenocene (200, 0.80)	Iodoruthenocene (172, 60)	4.83 (t, <i>J</i> = 2, 2 H, RcH), 4.57 (s, 5H, RcH), 4.46 (t, <i>J</i> = 2, 2 H, RcH)	Light yellow
Osmocene (200, 0.63)	Iodoosmocene (211, 74)	4.98 (d, <i>J</i> = 1.4, 2 H, OcH), 4.75 (s, 5H, OcH), 4.60 (d, <i>J</i> = 1.4, 2 H, OcH)	Light yellow

2.5.9 Synthesis of aminometallocenes

Copper iodide (6.0 mg, 0.032 mmol), Fe₂O₃ (5.0 mg, 0.032 mmol), aq. ammonia (25%, 1.5 mL) and NaOH (30 mg, 0.75 mmol) were added to a solution of the iodometallocene (100 mg) in ethanol (5 mL). The resulting mixture was heated at 90 °C for 6 h and subsequently cooled to room temperature. Diethyl ether (40 mL) was added and the ethereal solution was washed with phosphate buffer (pH 7.01, 10 mM, 10 mL). The organic phase was extracted three times with HCl (1 M, 10 mL), the pH of the aqueous extract adjusted to between 7-8 using aq NaOH (1 M), and then extracted three times with dichloromethane (10 mL). The combined organic extracts were dried over MgSO₄, filtered and the solvent removed in vacuo to yield the product.

Table 2.3. Experimental and characterisation data for the aminometallocenes **4a-c**.

	Yield (mg, %)	¹ H NMR (ppm)	ESI-MS (m/z)	Colour
4a	35, 54	4.10 (s, 5H, FcH), 3.99 (s, 2H, FcH), 3.84 (s, 2H, FcH), 2.59 (br, 2H, NH)	-	Orange
4b	40, 57	4.56 – 4.55 (m, 2H, R ₂ CH), 4.50 (s, 5H, R ₂ CH), 4.26 – 4.25 (m, 2H, R ₂ CH), 2.55 (br s, 2H, NH)	248 [M] ⁺	Dark yellow
4c	59, 80	4.83 (t, <i>J</i> = 1.4, 2H, OCH), 4.66 (s, 5H, OCH), 4.43 (d, <i>J</i> = 1.4, 2H, OCH), 2.50 (br s, 2H, NH)	336 [M] ⁺	Light pink

2.5.10 Coupling reaction for ebselen derivatives

A sample of **3a** dissolved in dry dichloromethane (5 mL) was added dropwise over 5 min to a stirred solution of the aminometalocene in dry dichloromethane (5 mL) at room temperature. The reaction was monitored by TLC and when completed (~6 h), the solvent was evaporated in vacuo, water (15 mL) was added and the mixture stirred for 2 h. The reaction mixture was filtered and the residue was recrystallized from methanol (**1a**), diethyl ether (**1b**) or dissolved in methanol and purified by TLC with ethyl acetate/hexane (1:1, v/v) (**1c**).

Table 2.5. Experimental and characterisation data for compounds **1a**, **1b**, **1c**.

	3a (mg, mmol)	Amino- metallocene (mg, mmol)	Yield (mg, %)	¹ H NMR (ppm)	ESI-MS (m/z)	Colour
1a	100, 0.50	4a (300, 1.50)	120, 61	8.03 – 7.42 (m, 4H, ArH), 4.88 (s, 2H, FcH), 4.29 (s, 5H, FcH), 4.17 (s, 2H, FcH)	384 [M] ⁺	Brown
1b	90, 0.35	4b (200, 0.80)	20, 39	7.98 (d, J = 8.2, 1 H, ArH), 7.60 (t, J = 8.2, 1 H, ArH), 7.53 (d, J = 7.8, 1 H, ArH), 7.40 (t, J = 7.8, 1 H, ArH), 5.32 (t, J = 1.8, 2 H, RcH), 4.70 (s, 5 H, RcH), 4.57 (t, J = 1.8, 2 H, RcH)	430 [M] ⁺	White

1c	90, 0.35	4c (200, 0.60)	21, 10	7.98 (d, $J = 7.4$, 1 H, ArH), 7.60 (t, $J = 7.4$, 1 H, ArH), 7.51 (d, $J = 7.8$, 1 H, ArH), 7.39 (t, $J = 7.8$, 1 H, ArH), 5.39 (t, $J = 1.4$, 2 H, OcH), 4.89 (s, 5 H, OcH), 4.73 (t, $J = 1.4$, 2 H, OcH).	518 [M] ⁺	Yellow
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2.5.11 Synthesis of compound 2

A solution of **3b** (100 mg, 0.5 mmol) dissolved in dichloromethane was added dropwise over 5 min to a stirred solution of **4a** (300 mg, 1.5 mmol) in dichloromethane in an ice/salt bath. The reaction was stirred for 1 h and the reaction progress was monitored by TLC. When the reaction is complete, the solvent was evaporated in vacuo and the residue was treated with water and stirred for 16 h. The mixture was filtered, and the residue was recrystallised from ethyl acetate, yielding compound **1a'** as orange crystals. Yield = 90 mg (54%)

¹H NMR (500MHz) δ 8.03 – 7.39 (m, 4H, ArH), 4.88 (s, 2H, FcH), 4.29 (s, 5H, FcH), 4.20 (s, 2H, FcH). ESI-MS, $m/z = 336$ [M]⁺

2.5.12 DTNB reduction by mammalian TrxR assay.

The experiment was performed in a reaction mixture (198 μL /well) containing TE buffer, TrxR (10 nM), BSA (0.1 mg/mL) and NADPH (4 mg/mL). Aliquots (2 μL) of the compounds were added into each well and incubated at 37 °C for 15 min. Activity of TrxR reducing DTNB by NADPH was measured at 412 nm for 5 min after the addition of 62.5 mM DTNB (7 μL).

2.5.13 NADPH consumption by mammalian TrxR assay.

A reaction mixture containing TE buffer, TrxR (10 nM), BSA (0.1 mg/mL) and NADPH (40 $\mu\text{g/mL}$) was prepared. Aliquots (2 μL) of the compounds were added into each well followed by the addition of reaction mixture (198 μL /well). NADPH consumption was measured immediately at 340 nm for 15 min.

2.5.14 NADPH consumption by GR assay.

The experiment was performed in a reaction mixture (198 μL /well) containing TE buffer, GR (2 nM), BSA (0.1 mg/mL) and NADPH (40 $\mu\text{g/mL}$). Aliquots (2 μL) of the compounds were added into each well and incubated at 37 °C for 15 min. GSSG (20 μL , 10mM) was added and measured at 340 nm for 5 min.

2.5.15 Interaction of compounds with GSH.

Aliquots (1 μL) of the compounds (5-25 μM) were added into each well containing GSH (600 μM) in TE buffer and incubated at 37 °C for 15 min. DTNB (12.5 μL , 20 mM) was added and measured at 412 nm for 5 min.

2.5.16 Cell Proliferation Assay

Stock solutions of compounds **1a-b** (100mM), ebselen (100mM) and $\text{Os}_3(\text{CO})_{10}(\text{ACN})_2$ (20mM) were prepared in sterile-filtered dimethyl sulfoxide (DMSO). These were then used to prepare working solutions with DMEM and DMSO that were 250 μM in compound and 2.5% v/v in EtOH (or DMSO). Serial dilutions from these were performed using 2.5% DMSO in serum-free DMEM.

The anti-proliferative activity of the compounds on MCF-7 was screened using MTS assay. Cells were first seeded in a 96-well plate with growth medium (DMEM with FBS) at the same initial density of 1×10^4 cells per well, and allowed to adhere and grow for 18h. Sterile water (100 μL) was added to the wells at the perimeter to prevent evaporation of the media from the enclosed wells. The cells were then serum-starved in serum-free DMEM (80 μL) for 6h, followed by treatment with 20 μL of the compound solutions prepared as above for 24 h; final treatment concentrations of compounds were in 0.5% DMSO in serum-free DMEM. Control cells were treated with vehicle (0.5% DMSO in serum-free DMEM).

2.5.17 Crystallographic Analysis

Crystals of **1a** and **2** were grown by slow evaporation of diethyl ether solutions. The crystals chosen were mounted on a quartz fiber. X-ray diffraction data were collected on a Bruker X8 APEX Single Crystal X-ray Diffractometer at 223(2) K, using Mo $\text{K}\alpha$ radiation, with the SMART suite of programs.²⁹ Data was processed and corrected for Lorentz and polarization effects with SAINT and for absorption effects with SADABS.³⁰ Hydrogen atoms were placed in calculated positions and refined with a riding model. Appropriate restraints were placed on all disordered parts. Structural solution and refinement were performed with SHELXTL suite of

programs.³¹ The structures were solved by direct or Patterson methods to locate the heavy atoms, followed by successive difference maps for the light, non-hydrogen atoms.

Table 2.5 Crystal and refinement data.

Compound	1a	2
Chemical formula	C ₁₇ H ₁₃ FeNOSe	C ₁₇ H ₁₃ FeNOS
Formula weight	382.09	335.19
Temperature, K	103(2)	203(2)
Wavelength, Å	1.54178	0.71073
Crystal size, mm x mm x mm	0.010 x 0.030 x 0.040	0.180 x 0.220 x 0.380
Crystal habit	red plate	red block
Crystal system	monoclinic	monoclinic
Space group	P 2 ₁ /c	P 2 ₁ /n
a, Å	a = 9.2280(4)	6.5229(5)
b, Å	b = 13.3493(5)	18.3576(14)
c, Å	c = 11.5586(5)	11.9118(11)
β , deg	β = 101.236(4)	90.340(2)
Volume, Å ³	1396.58(10)	1426.4(2)
Z	4	4
Density (calculated), g/cm ³	1.817	1.561
Absorption coefficient, mm ⁻¹	11.604	1.200
F(000)	760	688

2.5.18 DFT Calculations

DFT calculations were performed with the Gaussian 16A suite of programs, utilizing the hybrid functional of Truhlar and Zhao (M06).³² The Def2TZVP basis set, along with f- type polarization functions were employed for all atoms. Spin-restricted calculations were used for geometry optimization. Harmonic frequencies were then calculated to characterize the stationary points as equilibrium structures with all real frequencies, and to evaluate zero-point energy (ZPE).

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Chapter 3. Inhibition of TrxR by Triosmium Clusters

3.1 Cytotoxic Triosmium Carbonyl Clusters

In recent studies, osmium containing compounds have been found to be cytotoxic towards cancer cells.¹ These compounds includes osmium carbonyl clusters,^{1,2} and osmocenyl derivatives of a commercial drug, tamoxifen.³⁻⁵ Osmium carbonyl clusters such as **5** have been shown to exhibit high cytotoxicity for both ER+ and ER- breast cancer cells (Figure 3.1).^{1,2} The mode of action for their anti-proliferative effects remains unclear, although studies have shown that it interacts with intracellular -SH, -COOH and -CONH₂ residues.^{2,6-8} More recently, it was also shown that tamoxifen-like metallocifens, such as, osmocifen inhibit TrxR by interaction with its Sec residue, thereby leading to cell imbalance and eventual cell death.³ It is thus possible that **5** may act in a similar way, i.e., interaction with the selenide (Se⁻) on the Sec residue of TrxR.

In the course of related work with osmium carbonyl clusters derived from **5**, we have also found that clusters **6-7** were cytotoxic and apoptosis-inducing,¹ suggesting that these clusters may also have similar biological activity. Our studies on the reactivity of clusters **5-7** with simple molecular mimics of TrxR and other potential reactive moieties of TrxR, their cytotoxicity to MDA-MB-231 and MCF-7 cells, as well as their inhibitory effects on mammalian TrxR, are discussed in this chapter.

Figure 3.1. Molecular structures of clusters **5-7**.

3.2 Cytotoxicity and Reactivity Studies

3.2.1 Cytotoxicity assays with MDA-MB-231 and MCF-7

The anti-proliferative activity of the compounds on MDA-MB-231 was determined using the MTS assay (Table 3.1). Serum-free media were used during the incubation since clusters **5-7** are known to interact with FBS.² It is found that **6b** and **7** exhibit similar toxicity, and that **6a** has a higher IC₅₀, with both ER+ and ER- breast cancer (MCF-7 and MDA-MB-231 respectively) cells. These findings are consistent with prior studies.^{1, 6}

Table 3.1. Cell viability studies of MDA-MB-231 and MCF-7 with clusters **5-7**.

Compounds	MDA-MB-231 IC ₅₀ / μM	MCF-7 IC ₅₀ / μM
5	6.04 $\pm$ 0.15	8.10 $\pm$ 0.80
6a	6.77 $\pm$ 0.78	8.04 $\pm$ 0.51
6b	4.50 $\pm$ 0.41	6.14 $\pm$ 0.18
7	3.07 $\pm$ 0.28	5.04 $\pm$ 0.35

It is probable that a change in the bonding mode of the thiolate to an η^1 -SR form, thereby creating a vacant site on the osmium centre, is favored in **6b** and **7** via stabilization of the sulfur lone pair through delocalization into the phenyl group (Scheme 3.1). The availability of a vacant site on the metal centre would allow for interaction with biological targets such as an Se⁻. In contrast, the methylene group in between the sulphur atom of thiolate and the phenyl ring in **6a** prevented this stabilization.

Scheme 3.1. Stabilization of the η^1 -SR bonding mode.

3.2.2 Reactivity with simple molecular mimics of relevant biomolecules

The selenyl sulphide (S-Se) bond in the active site of TrxR can be reduced under physiological conditions to a thiol (SH) and a selenide (Se^-) moiety. Another ubiquitous functional group present in cells is the carboxylic acid (COOH) group, which is also known to react well with many osmium carbonyl clusters.⁹ The organic molecules (Se^- , SH, COOH and SeS) are used as molecular mimics for these functional groups (Table 3.2). The room temperature reactions of clusters **5-7** with the organic molecules were monitored through their metal-carbonyl vibrational modes (2100 – 1800 cm^{-1}) using FT-IR spectroscopy (Figures B8-B12 (appendix)). Only selenide (Se^-) reacted with all four clusters, and **5** reacted with all four molecular mimics. The reactivity study suggests that clusters **5-7** can all interact with organoselenides and hence the Se^- moiety in reduced TrxR.

Table 3.2. Reactivity of **5-7** with molecular mimics.

Compounds	Se^-	SH	COOH	SeS
5	✓	✓ *	✓ *	✓
6a	✓	✗	✗	✗
6b	✓	✗	✗	✗
7	✓	✗	✗	✗

*From reference ².

3.3 Activity of clusters 5-7 towards mammalian TrxR

3.3.1 DTNB Reduction

DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) is a substrate that is reduced directly by mammalian TrxR and NADPH to TNB^{2-} (Scheme 3.2). Inhibition of TrxR will thus lead to a reduction in the amount of TNB^{2-} generated, which can be measured by its absorption at 412 nm (A_{412}). A low gradient for the $[\text{TNB}^{2-}]$ vs time curve implies inhibited TrxR activity.

Scheme 3.2. Reduction of DTNB by mammalian TrxR and NADPH.

The inhibitory activity of clusters **5-7** for mammalian TrxR was determined, and the results show that **5** is an inhibitor (Figure 3.2a), with an IC_{50} of $5.3 \pm 0.9 \mu\text{M}$ for 10 nM of mammalian TrxR (Appendix B13). In contrast, clusters **6a-b** and **7** do not show any inhibitory activity, just as for the controls (vehicle and ebselen) (Figure 3.2b). This is in contrast with the reactivity studies above.

Figure 3.2. Measurements of DTNB (5,5'-dithiobis-(2-nitrobenzoic acid) reduction by NADPH catalyzed by TrxR at A₄₁₂ in TE buffer, TrxR (10 nM), BSA (0.1 mg/mL), NADPH (4 mg/mL) and DTNB (2.2 mM). Left (a) for Compound **5**, right (b) for compounds **6** and **7**.

3.3.2 NADPH consumption

The reduction of a substrate by TrxR is accompanied by the consumption of NADPH, which can be measured at 340 nm (A₃₄₀). The clusters were found not to be substrates of TrxR as their A₃₄₀ decreased slowly (for **5**) or not at all (for **6** and **7**), compared to the rapid decrease for ebselen, a known substrate (Figure 3.3).¹⁰

Figure 3.3. Measurements of NADPH consumption at A_{340} in TE buffer, TrxR (10 nM), BSA (0.1 mg/mL), and NADPH (40 μ g/mL).

3.4 Selectivity studies on **5**

3.4.1 NADPH consumption by glutathione reductase (GR)

TrxR is a homologue of glutathione reductase (GR); the main difference between these two enzymes is that the active site of GR is a cysteine residue whereas that of TrxR is a selenocysteine residue in a C-terminal extension not found in GR.¹¹ It was therefore interesting to find if **5** interacts selectively with TrxR but not GR. The interaction of **5** with GR was examined by measuring NADPH consumption in the glutathione system (Figure 3.4). The results show that NADPH was consumed by GR (A_{340} decreased rapidly), indicating that **5** did not interact with GR.

Scheme 3.3. NADPH consumption by GR, measured at A_{340} .

Figure 3.4. NADPH consumption assay of **5** in GR. Measurements of NADPH consumption were made at 340 nm (A_{340}), with TE buffer, GR (10 nM), BSA (0.1 mg/mL), GSSG (1 mM) and NADPH (40 μ g/mL).

3.4.2 Interaction with reduced glutathione (GSH)

Since the intracellular concentration of TrxR is in the nanomolar range while that of reduced glutathione (GSH) is up to the millimolar range, it is possible that the inhibitory effects of **5** with TrxR can be disrupted by GSH. As GSH reduces DTNB to form TNB^{2-} which absorbs at 412 nm, the amount of GSH remaining after incubation with **5** can be followed.

Scheme 3.4. Reduction of DTNB by GSH to form TNB²⁻.

The A₄₁₂ decreased with decreasing concentration of TNB²⁻ formed (scheme 3.4) after incubation of **5** (25 μ M, 10 μ M and 5 μ M respectively) with GSH (100 μ M) for 15 min, indicating that **5** interacted with GSH. This is not surprising since **6** and **7** were synthesized from the reaction of **5** with organothiols. This is, however, in contrast with the results from Kong *et al.*², although the author did not clearly specify the reduced or oxidised form of glutathione.

Figure 3.5. Measurement of DTNB reduction by GSH after incubating **5** with GSH (100 μ M) in TE buffer, followed by the addition of DTNB (1.8 mM) in EtOH.

3.4.3 Selectivity for mammalian TrxR over GSH

The reactivity of cluster **5** with GSH in the presence of TrxR was investigated by measuring NADPH consumption in the thioredoxin (Trx) insulin assay (scheme 3.5). This

assay is based on the reduction of insulin by Trx, coupled with the reduction of Trx by TrxR, with NADPH as the reducing agent.

Scheme 3.5. Thioredoxin (Trx) insulin system.

The Trx insulin assay on a sample in which **5** (10 μ M) was incubated with a mixture of TrxR (10 nM) and GSH (50 μ M, 500 μ M and 5 mM, respectively) showed that the amount of NADPH remained constant (Figure 3.6), indicating that **5** was still able to inhibit TrxR in the presence of excess GSH. No decrease in NADPH was observed across all concentration because TrxR has been inhibited by **5** with all concentrations of GSH, suggesting **5** to be selectively towards TrxR over GSH.

Figure 3.6. Measurements of NADPH consumption at A₃₄₀ after incubating cluster **5** (10 μ M) with TrxR (10 nM), NADPH (40 μ g/mL) and GSH (50 μ M, 500 μ M and 5 mM respectively) in TE buffer, followed by addition of Trx (1 μ M) and insulin (160 μ M).

3.4.4 Effect on TrxR enzymatic activity in MCF-7 cell lysates

The insulin endpoint assay measures the reduction of insulin as described in scheme 3.5. Instead of measuring NADPH consumption, the amount of reduced insulin generated was measured through the conversion of added DTNB to TNB²⁻. Using MCF-7 cell lysates after 15 mins incubation, **5** is shown to inhibit TrxR activity (Figure 3.7). However, serum-free media must be used during treatment of cells as **5** is known to react with BSA. This suggests inhibition of TrxR as one of the plausible apoptotic pathways.

Figure 3.7. Measurement of the generated sulfhydryl group on reduced insulin at A₄₁₂ in cell lysate (200 µg/mL), Tris-HCl (40 mM), Trx (500 nM), insulin (92 µM), NADPH (650 µM) and EDTA (6.25 mM).

3.5 Conclusion

In this study, it was found that the osmium clusters **5-7** interacted with the (Se⁻) moiety. Although all four clusters were cytotoxic, only **5** inhibited mammalian TrxR, with an IC₅₀ of 5.3 ± 0.9 mM, but it was not a substrate of TrxR. The TrxR inhibition of **5** was selective over GR, and the presence of excess GSH did not interfere with its inhibitory effects. That it also inhibited TrxR in cell lysates suggested that inhibition of TrxR is a potential apoptotic pathway. The clusters are known to interact with FBS, modification of the ligands is necessary and may be a promising approach in designing a triosmium carbonyl cluster for clinical applications.

3.6 Experimental

3.6.1 General Experimental.

Clusters **5-7** were synthesized according to literature methods and characterized by ¹H NMR and FT-IR spectroscopy.^{12, 3} NADPH and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma. Mammalian TrxR with approximately 14 U/mg was purchased from IMCO Ltd. Ebselen, clusters **5** and **6** were freshly dissolved in DMSO, and **7** in EtOH, prior to use. TE buffer (50 mM Tris-HCl pH 7.5, 2mM EDTA) was prepared by diluting 1 mM Tris-HCl and 0.2 M EDTA in deionized (DI) water. Stock solutions were prepared as follows: 62.5 mM DTNB in EtOH; BSA (10 mg/mL) in DI water; TrxR (1 mM), NADPH (40 mg/mL), GSH (100 mM), GSSG (10 mM) and Trx (20 μM), respectively, in TE buffer. Each bioassay was analyzed in triplicates and the values were obtained from at least two separate experiments with the mean and standard error calculated. The IC₅₀ for TrxR inhibition was determined from a sigmoidal dose response (variable slope) equation using the GraphPad Prism 7 software.

3.6.2 Reaction of clusters with phenylselenide.

Phenylselenol (10.6 μ L, 0.1 mmol) was dissolved in dry THF (3 mL) and NaH (2.4 mg, 0.01 mmol) was added. Bubbling was observed, and the solution was stirred at room temperature for 15 min. A solution of **5**, **6a-b** or **7** (0.01 mmol) in dry THF (3 mL) was then added into this phenylselenide solution. The reaction was monitored by using FT-IR spectroscopy. ^1H NMR spectra of the crude products were obtained after removal of solvent.

3.6.3 Reaction of clusters with phenylthiol, benzoic acid and diphenylselenysulfide.

Cluster **5**, **6a-b** or **7** (0.01 mmol) and phenylthiol (10.6 mL, 0.100 mmol) were dissolved in dry THF (5 mL) and stirred at room temperature. The reaction was monitored by using FT-IR spectroscopy. The same procedure was used for the reaction of **5**, **6a-b** or **7** with benzoic acid (COOH) and diphenylselenysulphide (S-Se) respectively.

3.6.4 Cell viability assays.

Stock solutions (20 mM) were prepared in sterile-filtered ethanol (EtOH). These were then used to prepare working solutions with the culture media and EtOH that were 250 μ M in compound and 2.5% v/v in EtOH. Cells were seeded in a 96-well plate with the culture media at the same initial density of 10 000 cells per well and allowed to adhere and grow before the media was replaced with serum-free media. The cytotoxicity of the compounds on MDA-MB-231 and MCF-7 was screened using MTS assay reagent.

3.6.5 DTNB reduction by mammalian TrxR in the presence of osmium clusters.

The experiment was performed in a reaction mixture (198 μ L/well) containing TE buffer, TrxR (10 nM), BSA (0.1 mg/mL) and NADPH (4 mg/mL). An aliquot (2.0 μ L) of compounds were added into each well and incubated at 37 $^{\circ}\text{C}$ for 15 min. Activity of TrxR reducing DTNB by NADPH was measured at 412 nm for 5 min after the addition of 62.5 mM DTNB (7.0 μ L).

3.6.6 NADPH consumption by mammalian TrxR in the presence of osmium clusters.

A reaction mixture containing TE buffer, TrxR (10 nM), BSA (0.1 mg/mL) and NADPH (40 µg/mL) was prepared. 2.0 µL of compounds were added into each well followed by the addition of reaction mixture (198 µL/well). NADPH consumption was measured immediately at 340 nm for 15 min.

3.6.7 NADPH consumption by GR in the presence of 5.

The experiment was performed in a reaction mixture (198 µL/well) containing TE buffer, GR (2 nM), BSA (0.1 mg/mL) and NADPH (40 µg/mL). An aliquot (2.0 µL) of compounds were added into each well and incubated at 37 °C for 15 min. GSSG (20 µL, 10 mM) was added and measured at 340 nm for 5 min.

3.6.8 Interaction of 5 with GSH.

An aliquot (1.0 µL) of the compounds was added into each well containing GSH (600 µM) in TE buffer and incubated at 37 °C for 15 min. DTNB (12.5 µL, 20 mM) was added and measured at 412 nm for 5 min.

3.6.9 Reactivity of 5 with GSH in the presence of TrxR in the insulin assay.

A reaction mixture containing TE buffer, TrxR (10 nM) and NADPH (40 µg/mL) was prepared. 2.0 µL of compound (10 µM) was added into each well, followed by the addition of GSH (100 µL, 0 – 5 mM). The reaction mixture (80 µL/well) was added and incubated at 37 °C for 15 min. TrxR activity was measured at 340 nm after adding a mixture (20 µL/well) of Trx (1 µM) and insulin (160 µM).

3.6.10 TrxR enzymatic activity in MCF-7 cell lysate determined by insulin endpoint assay.

MCF-7 cells were seeded into 6-well plate at the same initial density of 200 000 cells per well and allowed to adhere and grow for 24 h. Cells were washed with PBS (1 x 1mL) and serum-free medium were added. In each well, cells were treated with 2.0 mL of compound with 0.1 % DMSO as the vehicle and incubated for 3 h. Medium was removed from the wells and the cells were washed with PBS. Cells were then harvested with lysis buffer containing 50 mM Tris-HCl pH 7.5, 2 mM EDTA, protease inhibitor (50 times diluted) and 1 % triton. Freezing and thawing of the lysis mixture were repeated for 3 times followed by centrifugation at 4 °C, 15000 rpm 15 min. The protein concentration of the supernatant was determined by using DC Protein Assay. Incubation mixture containing Tris-HCl pH 7.5 (80 mM), Trx (1 µM), insulin (275 µM), NADPH (1.3 mM) and EDTA (12.5 mM) was prepared. 20 µL of cell lysate (200 µg/ml) was incubated with 30 µL of incubation mixture for 40 min at 37 °C. 200 µL of 1 mM DTNB in 6 M Gu-HCl was added to stop the reaction and the absorbance at 412 nm was recorded.

3.7 References

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Chapter 4. Reactivity of Dichalcogenides with Dinuclear Organometallic Complexes

4.1 Targeting the Active Site of Thioredoxin Reductase

As mentioned in Chapter 1, the active site of mammalian TrxR, including human TrxR (hTrxR), contains a sulphur-selenium (S-Se) bond across selenocysteine (Sec) and cysteine (Cys) residues in its oxidised form (Scheme 4.1).¹⁻³ This bond is unique as no other enzymes or proteins are known to contain this particular bond.⁴⁻⁶ Most of the drugs known to target TrxR exploit the high nucleophilicity of the selenide and thiol moieties in the reduced state, the major form in excess NADPH. Examples include ebselen and auranofin, which form covalent bonds with the selenide moiety of Sec and/or thiol moiety of Cys.⁷

Scheme 4.1. Redox of the TrxR active site.

This mode of action, however, suggests that they are not selective towards hTrxR since cysteines and selenocysteines are found in numerous other enzymes and proteins. For instance, the close homologue of S-Se moiety, the disulphide (S-S) bond, is ubiquitous in proteins. Reaction of a drug with S-S and/or Se(-) is probably responsible for many of the side-effects in chemotherapy.^{4, 7-12} Selective targeting of the S-Se bond is therefore highly desirable as it would equate to selectively targeting the active site of hTrxR. Distinguishing between the S-S and S-Se bonds is, however, difficult due to the small chemical difference between sulphur and selenium.³

One possible approach is to target the sulphur-selenium bond with a heterobimetallic compound. The slight differences in atomic properties between sulphur and selenium include:

- (a) Softness - selenium is softer than sulphur on Pearson's hardness scale,¹³
- (b) Electronegativity – there is a small difference in the electronegativities of sulphur and selenium; 2.58 and 2.55, respectively, on the Pauling scale,¹⁴
- (c) Covalent radius – the difference between the covalent radii of sulphur (1.05 Å) and selenium (1.20 Å) is significant.¹⁵

These differences in atomic properties will translate to differences between the S-S and S-Se bonds such as a slight dipole and a longer bond in the latter. A heterobimetallic species, with two different transition metal centres having similar chemical difference between them to that of the S-Se bond, may favour it over an S-S bond in chemical reactivity (Figure 4.1).

Figure 4.1. Schematic of heterobimetallic complex with S-Se bond, where M and M' are two different transition metals.

Human TrxR (hTrxR) is a relatively large protein, with a molecular weight of ~55000 amu. For the chemical reactivity studies, we settled upon the diphenyldichalcogenides as mimics of the

active site and disulfide bonds. Since the SeS bond in TrxR is part of an eight-membered ring system, we also examined 1,8-dichalcogenonaphthalenes as closed ring mimics (Figure 4.2).

E, E' = S or Se

Figure 4.2. Structures of the compounds **EE'** and **napEE'**.

The bimetallic system chosen for this study should exhibit the following properties:

- Reactive with dichalcogenides.
- Contain unique spectroscopic handles to allow monitoring of reactions.
- Commercially available or easy to synthesize.

The primary complex of interest identified was the heterobimetallic $(\eta^5\text{-C}_5\text{H}_5)(\text{CO})_2\text{RuMo}(\eta^5\text{-C}_5\text{H}_5)(\text{CO})_3$ (**RuMo**), the synthesis of which has been reported.¹⁶ The difference in the electronegativity for ruthenium (2.20) and molybdenum (2.16) is similar to that between sulphur and selenium. For comparison, the reactivity of the homometallic analogues $[(\eta^5\text{-C}_5\text{H}_5)\text{Ru}(\text{CO})_2]_2$ (**RuRu**) and $[(\eta^5\text{-C}_5\text{H}_5)\text{Mo}(\text{CO})_3]_2$ (**MoMo**) were also examined (Figure 4.3). Both **RuRu** and **MoMo** are known to react with **SS** and **SeSe**,¹⁷⁻¹⁹ and the cyclopentadienyl and carbonyl ligands provide unique spectroscopic handles through ^1H NMR and FTIR, respectively.

Figure 4.3. Molecular structures of **RuRu**, **MoMo** and **RuMo**.

4.2 Competitive reaction of RuRu with SS and SeS

Both **SS** and **SeSe** are commercially available, while **SeS** is easily prepared from phenylselenenyl bromide and thiophenol (Scheme 4.2).²⁰

Scheme 4.2 Reaction to prepare **SeS**.

Both the homobimetallic complexes **RuRu** and **MoMo** are commercially available. As the ruthenium complex **RuRu** is expensive and the reported synthesis lengthy and tedious, requiring freshly cracked cyclopentadiene²¹, we developed a microwave synthesis which generated the cyclopentadiene *in situ*; the entire process took 15 mins (Scheme 4.3).

Short lines denote CO ligands

Scheme 4.3 Microwave assisted synthesis of **RuRu**.

The heterobimetall complex **RuMo** can be prepared via the intermediate **RuI** (Scheme 4.4).¹⁶ The latter was in turn prepared from the reaction of **RuRu** with iodine in dichloromethane.²²

Scheme 4.4 Synthesis of **RuMo**.

As a preliminary study, the competitive reaction of equimolar amounts of **RuRu** with **SS** and **SeS** was carried out at 40 °C (Scheme 4.5).

Scheme 4.5 Competitive reaction of **RuRu** with **SS** and **SeS** (1:1:1 molar ratio) at 40 °C.

The concentrations of **RuRu**, **RuS** and **RuSe** were monitored by ¹H NMR spectroscopy via their Cp resonances and calculated against an internal standard. The concentrations of **SS** and **SeS** were derived on the assumption that the first reaction (equation 1) results in two moles of **RuS** and the second reaction (equation 2) affords equal amounts of **RuS** and **RuSe**, i.e., as given below:

$$\begin{aligned}
 [\text{SeS}]_t &= [\text{SeS}]_0 - [\text{reacted SeS}]_t \equiv [\text{SeS}]_0 - [\text{RuSe}]_t \\
 [\text{SS}]_t &= [\text{SS}]_0 - [\text{reacted SS}]_t \equiv [\text{SS}]_0 - \frac{[\text{RuS}]_t - [\text{RuS produced from (2)}]_t}{2} \\
 &\equiv [\text{SS}]_0 - \frac{[\text{RuS}]_t - [\text{RuSe}]_t}{2}
 \end{aligned}$$

In the first 10 hours of the reaction, the amount of **RuSe** formed was larger than that of **RuS**, which suggested that the reaction of **RuRu** with **SeS** was faster than its reaction with **SS**. The changes in concentrations of **SeS** and **SS** were also consistent with this. Beyond 10 h, the amount of **RuS** formed became larger than that of **RuSe**. This seemed to indicate that **RuRu** reacted faster with **SS** when the concentration of **SeS** was much less than **SS** (Figure 4.4).

Figure 4.4. Concentration vs time graph of the competitive reaction between **SS** and **SeS** with **RuRu** in a 1:1:1 molar ratio, where **[SS]** and **[SeS]** were derived.

The separate reactions of **RuRu** with **SS** and **SeS** under pseudo first-order conditions (10 equivalents of **SS** or **SeS**), however, showed that the reaction with **SS** was faster than that with **SeS**; the rate constants were determined to be 1.3 and 0.33 h^{-1} , respectively (Figure C8). This appears to contradict the results above and, in particular, it was observed that in the reaction with **SeS**, the amount of **RuS** produced was much lower than expected from the assumed stoichiometry of the reaction (Figure 4.5).

Figure 4.5. Graph of concentration of **RuRu**, **RuS** and **RuSe** for the pseudo first-order reaction between **SS** and **RuRu** (red line), and between **SeS** and **RuRu** (blue line).

These results suggest that the reaction with **SeS** may have resulted in the formation of the disulphide, i.e., **SS**, rather than **RuS**. To verify this, **RuRu** was reacted with three equivalents of **SeS^{Me}**; formation of the disulphide **S^{Me}S^{Me}** was identified by its methyl resonance in the ¹H NMR spectrum (Scheme 4.6 and Figure 4.6).

Scheme 4.6. Reaction of **RuRu** with **SeS^{Me}**.

Figure 4.6. ¹H NMR spectrum of the reaction of **RuRu** with three equivalents of **SeS^{Me}** at 50 °C, 21 h. Only the Cp (left) and methyl (right) regions are shown.

The methyl resonances of SeS^{Me} and $\text{S}^{\text{Me}}\text{S}^{\text{Me}}$ were coincident ($\delta_{\text{H}} = 2.32$ ppm) and so the amount of $\text{S}^{\text{Me}}\text{S}^{\text{Me}}$ formed was estimated in two ways:

- The concentrations of RuS^{Me} and RuSe were determined via their Cp resonances (at 5.37 and 5.36 ppm, respectively). The difference between these amounts would correspond to the amount of $\text{S}^{\text{Me}}\text{S}^{\text{Me}}$ formed.
- Since the amount of SeS^{Me} consumed equalled the amount of RuSe formed (28.7 μM), subtraction of the amount due to the unreacted SeS^{Me} (31.3 μM) from the peak intensity for the methyl resonance at 2.32 ppm (50.8 μM) would yield twice the concentration of $\text{S}^{\text{Me}}\text{S}^{\text{Me}}$ formed, as $\text{S}^{\text{Me}}\text{S}^{\text{Me}}$ has two methyl groups compared to one of SeS^{Me} .

The two methods gave calculated $[\text{S}^{\text{Me}}\text{S}^{\text{Me}}]$ of 11.1 and 9.8 μM , respectively, i.e., a difference of ~12% (Appendix C1). This confirmed the formation of a disulphide species in the reaction of RuRu with an excess of a selenyl sulphide species (Scheme 4.7), where the reaction of RuRu and SeS^{Me} in a 1:3 ratio gave $\text{S}^{\text{Me}}\text{S}^{\text{Me}}$, RuS^{Me} and RuSe in a ratio of 1.6:1:1.6. The formation of a disulphide species in this reaction accounts for why the concentration of SS remained almost unchanged in the course of the competitive reaction (Figure 4.4).

Scheme 4.7. Reaction of RuRu with SeS forming SS .

The formation of a disulphide suggested that radical species were involved. In order to verify this, a reaction of **RuRu** with **SS** (10 equivs) and TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy) (4 equivs); a commercially available radical scavenger, was carried out. The reaction rate dropped drastically; $k = 0.018$ vs 0.070 min^{-1} without TEMPO (Appendix C2).

To further confirm the formation of disulphides, and the plausible free radical reaction pathway, a heterodichalcogenide with different ^1H NMR handle on the S and Se fragment was prepared (Scheme 4.8).

Scheme 4.8. Preparation of $\text{Se}^{\text{OMe}}\text{S}^{\text{Me}}$

Reaction of compound **RuRu** with $\text{Se}^{\text{OMe}}\text{S}^{\text{Me}}$ saw the formation of $\text{S}^{\text{Me}}\text{S}^{\text{Me}}$ based on the ^1H NMR spectrum (Scheme 4.9); the formation of $\text{S}^{\text{Me}}\text{S}^{\text{Me}}$ was monitored through its CH_3 signal at 2.32 ppm. The difference in the formation of RuSe^{OMe} and RuS^{Me} could be accounted for by the formation of $\text{S}^{\text{Me}}\text{S}^{\text{Me}}$. The confirmation of the formation of disulphides in reactions involving **SeS** suggests that a free radical reaction mechanism may be involved.

Scheme 4.9. Reaction of **RuRu** with $\text{Se}^{\text{OMe}}\text{S}^{\text{Me}}$

That the reactions were light initiated was confirmed through two identical reactions of **RuRu** with **SS**, one placed in a clear NMR tube and the other in an amberised NMR tube, under visible light irradiation (30W, 3 min). It was found that the former reaction was significantly faster than the previous experiments with only ambient light irradiation, while no reaction was observed in the amberised NMR tube (Figure 4.7). A proposed reaction cycle for the competitive reaction is summarised in Scheme 4.10.

Figure 4.7. Graph of concentration vs time for the reaction of **RuRu** with **SS** in clear and amberised NMR tubes.

Scheme 4.10. Free radical cycle of the reaction of **RuRu** with **EE**.

4.3 Reaction under excess dichalcogenides

The realisation that the reactions involved the initial photogeneration of **Ru•** radicals prompted a re-analysis of the reactions carried out between **RuRu** with **EE** in excess. For the simpler reactions, i.e., with the homo-dichalcogenides, we have the photo-initiation step:

This can be considered as a fast pre-equilibrium, so that we have,

$$[\mathbf{Ru\bullet}] = 2c[\mathbf{RuRu}]; \text{ where } 0 \leq c \leq 1$$

Essentially, upon irradiation, dissociation into radicals is immediate and reaches a steady state. The constant *c* is a function of the quantum yield (ϕ), irradiation intensity, and the rate of radical recombination (reverse reaction). More importantly, there is no time dependence as the reaction only goes forward when the radicals are formed, and it shuts down completely in the absence of irradiation (fast radical recombination).

The organometallic radical **Ru•** can react in three ways:

Since **EE** is in excess, pathway (2 α) is likely to be dominant. In the case of **SeS**, both **Se•** and **S•** can be generated. Similarly, the radical **E•** can react in four ways, one of which is 2 γ above:

The radical termination pathways (2g and 3b) are of low probability as the radicals are in much lower concentrations. Eqn 3 γ has no effect on $[\mathbf{E}\bullet]$ except for the **SeS** reactions. With excess **EE**, eqn 2 α should be fast and therefore the rate determining step (rds) is likely to be eqn 3 α , giving:

$$-\frac{d[\mathbf{RuRu}]}{dt} = k_3[\mathbf{RuRu}][\mathbf{E}\bullet]$$

Since fast eqn 2 α is assumed, then $[\mathbf{E}\bullet] = [\mathbf{Ru}\bullet]$ and hence,

$$-\frac{d[\mathbf{RuRu}]}{dt} = 2ck_3[\mathbf{RuRu}]^2$$

and we have a second order rate law. Pseudo-second order reactions have been reported by others.²³⁻²⁸ The integrated form is:

$$1/[\mathbf{RuRu}] - 1/[\mathbf{RuRu}]_0 = (2ck_3)t$$

and a plot of $(1/[\mathbf{M-M}])$ vs t will have gradient $k_3' = 2ck_3$. The constant c is a function of ϕ and hence of **RuRu**, but is independent of **E**. On the other hand, k_3 is a function of both **RuRu** and **E**.

This means that for the two reactions, (**RuRu** + **SS**) and (**RuRu** + **SeSe**), for the same bimetallic complex, we have:

$$m^{S-S}/m^{Se-Se} = k_3^{S-S}/k_3^{Se-Se}$$

We now have a means to directly compare the rates (or rate constants) between those two reactions and draw conclusions about the relative reactivity of **RuRu** for **SS** over **SeSe**.

For reactions with **EE'** (where $E \neq E'$), eqn 3 γ can lead to:

and then,

$$-\frac{d[\mathbf{E\cdot}]}{dt} = k_3[\mathbf{RuRu}][\mathbf{E\cdot}] + k_4[\mathbf{EE'}][\mathbf{E\cdot}]$$

which under excess **EE'** implies that the second term will dominate and hence

$$-\frac{d[\mathbf{E\cdot}]}{dt} \approx k'_4[\mathbf{E\cdot}]$$

More importantly, it tells us that if eqn 3 γ is favoured (for $\mathbf{E\cdot} = \mathbf{S\cdot}$ or $\mathbf{Se\cdot}$) then it results effectively in the conversion of $\mathbf{E\cdot}$ to $\mathbf{E'\cdot}$ radicals. In other words, a fast reaction of **Ru**• with **E-E'** to give **RuE** may still result in **RuE'** as the major product if eqn 4 is favoured.

For the homobimetallic systems (**RuRu** and **MoMo**), the products are known (Scheme 4.11). The reactions carried out with the dichalcogenides in excess were monitored by ^1H NMR spectroscopy and measured in triplicates to ensure reproducibility (Figure 4.8, Appendix C3-5), and the rate constants determined from plots of $1/[\mathbf{MM}]$ vs time (Figure 4.9). For the reactions of **RuMo** which has two cyclopentadienyl ligands, the integration of both Cp resonances were equal and summed for computation of the concentration of **RuMo**.

Scheme 4.11. Reactions of **MM'** with **EE'**.

The results show clearly that **RuRu** has high preference for **SS** while **MoMo** has high preference for **SeS** and **SeSe**. For **RuMo**, it appears to follow the same trend as **RuRu** albeit at a significantly slower rate. This is consistent with the generation of both **Ru●** and **Mo●** radicals; since the reactions involving the former is significantly faster, the trend follows that of **RuRu**.

Figure 4.8. Graph of concentration vs time (Red) and 1/concentration vs time (Blue) for the reaction of **RuRu** with **SS**.

Figure 4.9. Plots of $1/[MM']$ vs time for the reaction of MM' with EE' . Red = RuRu, Blue = RuMo, Green = MoMo. Circle = SS, Square = SeSe, Triangle = SeS (left), histogram of the rate constants (right)

In reactions involving a homobimetallic with a homodichalcogenide, such as **RuRu** with **SS**, only the expected **ME** products were observed. In the reactions of **SeS**, however, it was found that its reaction with **RuRu** afforded **RuSe** in excess over **RuS** (Appendix C6) while its reaction with **MoMo** afforded **MoS** in excess over **MoSe** (Figure 4.10, Appendix C7). This is in apparent conflict with the observed rate constants; **RuRu** reacted significantly faster with **SS** while **MoMo** reacted faster with **SeSe**. These observations suggest that the **Ru•** radical prefers attack at the Se atom, and the **Mo•** radical prefers attack at the S atom, of the **SeS**. This hypothesis is also supported by a DFT calculation (Appendix C11) where **RuSe** and **MoS** are thermodynamically more stable products than **RuS** and **MoSe**, respectively. For the reaction of **RuMo** with **SeS**, the product distribution was: **MoS** > **RuSe** > **RuS** > **MoSe** which is consistent the observation as above (Appendix C10).

Figure 4.10. Plot of the formation of products in the reaction of **MoMo** with **SeS**.

4.4 Kinetics Study with 1,8-dichalcogenonaphthalenes

A similar set of reactions of **MM'** with 1,8-dichalcogenonaphthalenes (**napEE'**), in which the E-E' bond was within a cyclic system, was also carried out; the synthetic route to the dichalcogenides are depicted in Scheme 4.12 and representative kinetic plots for the reaction of **RuRu** with **SS** and **napSS** are given in Figure 4.11. The kinetics for **napEE'** generally obeyed the same trend as the linear molecules **EE'** albeit significantly (> 50-fold) slower (Appendix C12-13). No reaction was observed for reactions involving **RuMo**.

Scheme 4.12. Synthetic route to **napEE'**.

Figure 4.11. Plot of $1/[\text{RuRu}]$ for the reactions of **RuRu** with **SS** ($k_{\text{obs}} = 74.2 \text{ mM}^{-1} \text{ min}^{-1}$) and **napSS** ($k_{\text{obs}} = 1.10 \text{ mM}^{-1} \text{ min}^{-1}$).

It is postulated that the slower kinetics is due to the chelate effect; the S-centred radical that result from initial attack of the **Ru•** radical can either re-form **napSS** by kicking out the **Ru•** radical, or terminate via reaction with another **Ru•** radical to form **napRuSRuS** (Scheme 4.13). This chelate effect should be less pronounced on larger and/or less strained rings.

Scheme 4.13. Free radical mechanism involving **napSS**.

4.5 Conclusion

The reactions of the bimetallic species **RuRu**, **MoMo** and **RuMo** were found to proceed via a radical mechanism initiated by visible light irradiation. For reactions with an excess of the dichalcogenide, a second order rate law was derived, and analysis of the kinetic data showed that **RuRu** reacted faster with **SS**, while **MoMo** reacted faster with **SeSe**, and **RuMo** showed comparable rates for all three dichalcogenides. That **MoMo** reacted significantly faster with **SeS** than with **SS** suggested that it may be a good candidate for the selective targeting of TrxR. As these bimetallic species are not soluble in aqueous mediums, they were not tested for direct targeting of TrxR.

4.6 Experimental

4.6.1 General Experimental

All reactions and manipulations were carried out under an argon atmosphere using standard Schlenk techniques unless otherwise stated. Solvents that were used for reactions were distilled over the appropriate drying agents under argon before usage unless otherwise stated. **MoMo** was purchased from Merck. **RuMo** was synthesized according to literature.¹⁶ ¹H NMR spectra were recorded on a Joel ECA 400 or ECA 400SL at 400 MHz and referenced to the residual proton resonance of CDCl₃, unless otherwise stated. Internal standard use for kinetic studies is 1,3,5-trimethoxybenzene unless otherwise stated. Microwave assisted synthesis were performed on Anton Paar Monowave 400. Preparative TLC separations were carried out on Merck silica gel 60 Å F 254 20 x 20 cm² plates. All other reagents were used as received from commercial suppliers. The Cp proton resonances of all NMR tube kinetics reactions are tabulated in Appendix C12.

4.6.2 Microwave assisted synthesis of [Ru(η^5 -C₅H₅)(CO)₂]₂, RuRu

A mixture of Ru₃(CO)₁₂ (85 mg, 0.133 mmol) and dicyclopentadiene (1 ml, 7.41 mmol) were added to a 30 ml microwave synthesizer glass vial G30. Heptane (10 ml) was added to the mixture and the glass vial was fitted with a septum with a ruby thermometer probe adapter. The mixture was heated as fast as possible to 200°C in the microwave synthesizer, held at that temperature for 5 min and cooled to 55°C before it was removed. The glass vial was stirred in air for 5 min. The mixture was then heated as fast as possible to 200°C in the microwave synthesizer, held at that temperature for 10 min and cooled to 55°C before it was removed. The mixture was added to a short column of silica, washed with hexane and flushed out with DCM. The mixture was evaporated in vacuo to yield the product as orange crystals. Yield: 84 mg (95%)
IR (CH₂Cl₂, cm⁻¹): ν_{CO} 2003 (s), 1966 (s), 1934 (m), 1771 (s). ¹H NMR δ 5.28 (s, 10H, CpH).

4.6.3 Synthesis of SeS and SeS^{Me}

Phenylselenenyl bromide (151 mg, 0.64 mmol) was dissolved in DCM (10 mL). Pyridine (3 drops) was added, followed by the dropwise addition of a solution of the corresponding thiol (0.64 mmol) in DCM (3 mL). The reaction was left to stir at rt for 1 h. The reaction mixture was then concentrated under reduced pressure to afford a yellow oil. The yellow oil was dissolved in minimal amount of DCM and purified by preparative TLC using DCM-hexane (1:1) as the eluent. For SeS, yield: 92 mg (54 %), ¹H NMR δ 7.19 - 7.63 (m, 10H ArH), R_f = 0.70, Yellow oil; solidify upon standing in fridge.

For SeS^{Me}, yield : 79 mg (44 %), ¹H NMR δ 7.09-7.65 (m, 9H ArH), 2.34 (s, 3H, CH), R_f = 0.65, Yellow oil; solidify upon standing in fridge.

4.6.4 Synthesis of Se^{OMe}Se^{OMe}

Into a 50 mL, double-necked, round bottom flask were magnesium (65 mg, 2 mmol) in anhydrous diethyl ether (5 mL). A piece of iodine was added and the resulting mixture was heated at reflux until the solution turns colourless. 1-bromo-4-methoxybenzene (500 mg, 2 mmol) was added and the resulting mixture was heated at reflux over a period of 45 mins. The resulting pale-yellow solution was then cooled down to room temperature, selenium powder (210 mg, 2 mmol) was added, and the mixture brought to reflux for an additional 30 mins; the pale-yellow solution turned dark green on addition of the selenium. Excess Grignard reagent was hydrolysed by stirring the cooled mixture with water (0.1 mL). It was then cooled in an ice bath while bromine (65 μ l, 2 mmol) was added, followed by the slow addition of aqueous ammonium chloride (148 mg, 2 mmol, in 0.4 mL of water). The mixture was filtered, dried over anhydrous sodium sulphate, and the solvent removed under reduced pressure to afford an orange residue. This was separated via TLC

using an eluent of DCM/Hexane (10:90, v/v). The major yellow band ($R_f = 0.30$) was recovered and identified as compound **Se^{OMe}Se^{OMe}**. Yield = 73 mg (0.196 mmol, 9.8%).

^1H NMR: δ 7.54-7.52ppm (m, 4H, ArH), 6.82-6.79ppm (m, 4H, ArH), 3.82ppm (s, 6H, OCH₃).

4.6.5 Synthesis of **Se^{OMe}S^{Me}**

To a DCM (7 mL) solution of **Se^{OMe}Se^{OMe}** (73 mg, 0.196 mmol) placed in a 50 mL, double-necked, round bottom flask was added bromine (10 μL , 0.196 mmol) and a drop of pyridine. A solution of 4-methylthiophenol (48.6 mg, 0.392 mmol) in DCM (1.8 mL) and was added dropwise and the mixture was stirred at room temperature for an hour; the dark orange mixture turned pale yellow upon the addition of the thiol. The solvent was then removed under reduced pressure to afford an orange crude oil. This was separated via TLC using an eluant of DCM/Hexane (1:1, v/v). The yellow band (band 2, $R_f = 0.65$) was recovered and identified as compound **Se^{OMe}S^{Me}**. Yield = 18 mg (60 μmol , 15%). The major yellow band (band 1, $R_f = 0.40$) was recovered and identified as unreacted **Se^{OMe}Se^{OMe}**.

^1H NMR: δ 7.52-7.51ppm (d, 2H, $J = 4$ Hz, ArH), 7.50-7.49 (d, 2H, $J = 4$ Hz, ArH), 7.11-7.10 (d, 2H, $J = 4$ Hz, ArH), 6.82-6.79 (d, 2H, $J = 12$ Hz, ArH), 3.80ppm (s, 3H, OCH₃), 2.32ppm (s, 3H, CH₃).

4.6.6 Competitive One-pot Kinetics Experiment

A solution of **RuRu** (8.9 mg, 0.020 mmol) dissolved in chloroform-d (1 mL) was added to a vial containing the disulphide (0.020 mmol). The solution was thoroughly mixed and transferred to a vial containing the selenium-sulphide (0.020 mmol). Xylene (1 drop) was added to the mixture and the mass of the xylene added was recorded. The resulting solution was transferred into a schlenk NMR tube (0.5 mL). The solution was incubated in a 40°C water bath and removed from the water bath periodically for NMR experiments.

4.6.7 Preliminary Pseudo First-order Experiment

A solution of **RuRu** (8.9 mg, 0.020 mmol) dissolved in chloroform-d (1 mL) was added to a vial containing the disulphide or selenium-selenide (0.20 mmol). Xylene (1 drop) was added to the mixture and the mass of the xylene added was recorded. The resulting solution was transferred into a schlenk NMR tube (0.5 mL). The solution was incubated in a 40°C water bath and removed from the water bath periodically for NMR experiments.

4.6.8 Pseudo-Second-Order Experiment

For the kinetics studies, a solution of **MM'** (0.0100 mmol), **EE'** or **napEE'** (0.100 mmol) and the internal standard (1,3,5-trimethoxybenzene) in C₆D₆ (0.6 mL) were placed into an NMR tube and subjected to visible light irradiation, and the NMR spectra recorded periodically.

4.6.9 Synthesis of napEE'

In a two-neck rbf, magnesium (170 mg, 7.0 mmol) and a crystal of iodine was added into 20 mL of THF. The solution was refluxed until the solution turns colourless. The solution was cooled to rt before 1,8-dibromonaphthalene (850 mg, 3.0 mmol) was added and the solution was refluxed for 8 h. Elemental sulphur (224 mg, 7.00 mmol) was added and refluxed for 2h. The mixture was cooled to rt and quenched by addition of saturated NH₄Cl (10 mL). Air was bubbled into the reaction mixture for 2 h. The reaction mixture was diluted with DCM (20 mL) and the organic layer was separated. The organic layer was washed with water, brine and dried over MgSO₄. The solvent was removed to give a yellow crude oil. The crude product was purified by column chromatography with hexane as the eluent, and the product was recrystallised from ethanol to give the product as orange crystals. Yield = 399 mg (70 %)

^1H NMR (CDCl_3) δ 7.35 (d, $J = 8$ Hz, 2H, ArH), 7.27 (t, $J = 8$ Hz, 2H, ArH), 7.15 (d, $J = 8$ Hz, 2H, ArH)

4.6.10 DFT Calculations

DFT calculations were performed with the Gaussian 16A suite of programs,³⁴ utilizing the hybrid functional of Truhlar and Zhao (M06).²⁹ The Def2TZVP basis set, along with f- type polarization functions were employed for all atoms. Spin-restricted calculations were used for geometry optimization. Harmonic frequencies were then calculated to characterize the stationary points as equilibrium structures with all real frequencies, and to evaluate zero-point energy (ZPE).

4.7 References

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Chapter 5. Conclusion and Future Work

In this work, two approaches towards targeting TrxR with organometallic complexes were examined. In the first approach, two groups of organometallic complexes were designed and/or assessed as potential TrxR inhibitors, *viz.*, organometallic derivatives of ebselen or ebsulfur **1-2** and the triosmium carbonyl clusters **5-7** (Figure 5.1). The first group comprises organometallic derivatives of compounds which have been shown to be substrates of TrxR, while the second group is known to be cytotoxic against cancer cells via the metal centres.

Figure 5.1. Organometallic derivatives **1-7** examined as TrxR inhibitors.

Among these complexes, it was found that **1** and **5** were competitive (against Trx) and non-competitive inhibitors, respectively, of TrxR. While the metallocenyl derivatives of ebselen displayed superior activity towards TrxR compared to the parent molecule and Trx, the precise role of the metallocenyl moiety remains unclear. No appreciable structural difference between their bond parameters was observed, suggesting that the difference in activity was likely electronic and likely related to redox properties in the group VIII metal centre of the metallocene. Elucidation of this merits further study but the idea of synergy between an organic molecule that can act as a substrate to TrxR with a redox-active organometallic fragment appears to be a good way forward towards further development of this approach as it represents a simple yet effective way of

introducing redox properties to organic molecules. With the triosmium carbonyl cluster **5**, TrxR inhibition was clearly related to the presence of the labile ACN ligand. Its poor solubility in water as well as its interactions with serums, however, remains a problem towards its development into a drug. Modifications to impart greater solubility and bioavailability would be desirable. A typical strategy would be to introduce ligands such as -COOH to improve the solubility.

In the second approach, the chemistry of the Se-S bond, which is a unique characteristic of the active site of TrxR, with organometallic complexes was examined. It was centered around the idea that the Se-S bond may be selectively targeted by a heterobimetallic complex such as **RuMo**; the Se-S bond of TrxR was represented by two simple mimics, *viz.*, **EE'** and **napEE'** (Figure 5.2).

Figure 5.2. The heterobimetallic complex **RuMo** and the TrxR mimics **EE'** and **napEE'**.

Detailed kinetic studies, mostly based on the homobimetallic analogues, showed that their reactions with the E-E' bonds proceeded through initial photo-cleavage of the M-M' bond to form organometallic radicals. More importantly, the reactivity studies revealed that the two chalcogens had differing affinity for the two metals. In essence, the ruthenium radical had a greater affinity for the selenium atom while the molybdenum radical had a greater affinity for the sulphur atom, of the selenyl-sulphide bond (Scheme 5.1); this was counter to the case with the homodichalcogenides and suggested the possibility of selectively targeting TrxR with hetero-multimetallic complexes. Photoinitiation opens the possibility of photodynamic therapy, although

it may also be desirable to avoid the generation of radicals. Organometallic complexes that may react via reaction pathways which do not involve free radicals should be explored. One possibility would be complexes containing labile ligands; the other would be multiply metal-metal bonded complexes.^{1,2} These systems should be tested for their activity towards TrxR as well.

Scheme 5.1. Affinity of Ru and Mo to Se and S.

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Chapter 2

Dose Response Curve of 1a Against MCF-7

A1. Dose response curves (MTS) of **1a** (μM) on MCF-7.

Dose Response Curve of 1b Against MCF-7

A2. Dose response curves (MTS) of **1b** (μM) on MCF-7.

Dose Response Curve of Ebselen Against MCF-7

A3. Dose response curves (MTS) of ebselen (μM) on MCF-7.

Dose Response Curve of 5 Against MCF-7

A4. Dose response curves (MTS) of 5 (μM) on MCF-7.

A5. DTNB reduction assay and dose response curve of **1a** on TrxR. TrxR were incubated with different concentrations of the compound **1a** in presence of 400uM of NADPH (4 mg/mL). After 15 minutes, 2.2 mM of DTNB was added and the absorbance of the samples was recorded at 412nm every 10s for 5 min.

Inhibitory Response curve of [1a] on 10nM hTrxR

A6. DTNB dose response curve for **1a** (μ M) on TrxR.

Inhibitory Response curve of [1b] on 10nM hTrxR

A7. DTNB dose response curve of **1b** (μM) on TrxR.

A8. NADPH consumption assay for **1a**. Measurements of NADPH consumption were made at 340 nm (A_{340}), with TE buffer, TrxR (10 nM), BSA (0.1 mg/mL), and NADPH (40 $\mu\text{g/mL}$). The compound is substrate of TrxR as their A_{340} decreased rapidly, compared to the rapid decrease for ebselen, a known substrate.

A9. Lineweaver-Burk plot for **1a** (orange) and DTNB (control, blue). 50 nM of TrxR, 0.50 mL of 50 mM Tris-Cl, 1 mM EDTA, 250 μ M NADPH at pH 7.5, was added 1 to 10 μ M of compound **2b**, and absorbance at 340nm was followed against an identical blank without the TrxR.

A10. Lineweaver-Burk plot for **1b** (orange) and DTNB (control, blue). 50 nM of TrxR, 0.50 mL of 50 mM Tris-Cl, 1 mM EDTA, 250 μ M NADPH at pH 7.5, was added 1 to 10 μ M of compound **2b**, and absorbance at 340nm was followed against an identical blank without the TrxR.

A11. NADPH consumption of **1a** in GR assay. Measurements of NADPH consumption were made at 340 nm (A_{340}), with TE buffer, GR (10 nM), BSA (0.1 mg/mL), and NADPH (40 $\mu\text{g/mL}$).

A12. Measurement of DTNB reduction by GSH after incubating **1a** with GSH (100 μM) in TE buffer, followed by the addition of DTNB (1.8 mM) in EtOH.

Chapter 3

Dose Response Curve of 5 Against MDA-MB-231

B1. Dose response curves (MTS) of **5** (μM) on MDA-MB-231.

Dose-response curve of 6a on MCF-7

B2. Dose response curves (MTS) of **6a** (μM) on MCF-7.

Dose-response curve of **6a** on MDA-MB-231

B3. Dose response curves (MTS) of **6a** (μM) on MDA-MB-231.

Dose-response curve of **6b** on MCF-7

B4. Dose response curves (MTS) of **6b** (μM) on MCF-7.

Dose-response curve of **6b** on MDA-MB-231

B5. Dose response curves (MTS) of **6b** on MDA-MB-231.

Dose-response curve of **7** on MCF-7

B6. Dose response curves (MTS) of **7** on MCF-7.

Dose-response curve of **7** on MDA-MB-231

B7. Dose response curves (MTS) of **7** on MDA-MB-231.

B8. FTIR spectra of clusters **5** with **Se⁻**.

B9. FTIR spectra of clusters **6a** with Se⁻.

B10. FTIR spectra of clusters **6b** with Se⁻.

B11. FTIR spectra of clusters **7** with **Se⁻**.

B12. FTIR spectrum of cluster **5** with **SeS**

Dose-response curve of **5** on DTNB Reduction Assay

B13. Dose-response curve of **5** on DTNB reduction assay

Chapter 4

δ_H (ppm)	Compound	Proton signal	Integration intensity	Concentration ($\mu\text{mol/L}$)
5.37	RuS^{Me}	CpH	1.66	6.6
5.36	RuSe	CpH	7.17	28.7
2.32	S^{Me}S^{Me} + SeS^{Me}	-CH ₃	7.62	50.8
2.30	Xylene	-CH ₃	6.00	20.0
2.26	RuS^{Me}	-CH ₃	0.98	6.5

Method (a):

Assume that the difference between the amounts of **RuS^{Me}** and **RuSe** formed are converted to **S^{Me}S^{Me}**

Difference between [**RuSe**] and [**RuS^{Me}**]: $28.7 - 6.6 = 22.1 \mu\text{mol/L}$

Amount of unreacted **S^{Me}•**: $22.1 \mu\text{mol/L}$

[**S^{Me}S^{Me}**] formed: $\frac{22.1}{2} = 11.1 \mu\text{mol/L}$

Method (b):

Assume that the methyl resonance is due to **S^{Me}S^{Me}** and **SeS^{Me}**, where the amount of **SeS^{Me}** consumed in the reaction is equal to the amount of **RuSe** formed

Unreacted [**SeS^{Me}**]: $60.0 - 28.7 = 31.3 \mu\text{mol/L}$

[**S^{Me}S^{Me}**] formed: $\frac{50.8-31.3}{2} = 9.8 \mu\text{mol/L}$

% difference between the calculated [**S^{Me}S^{Me}**]: $\frac{11.1-9.8}{11.1} \times 100\% = 12\%$

C1. Calculation of [**S^{Me}S^{Me}**] formed

C2. Reaction of **RuRu** with **SS**, TEMPO vs control

C3. Reaction of **RuRu** with **EE** (conc vs time)

C4. Reaction of MoMo with EE (conc vs time)

C5. Reaction of RuMo with EE (conc vs time)

C6. Product distribution RuRu with SeS

C7. Product distribution MoMo with SeS

C8. Product Distribution RuMo with SS

C9. Product Distribution RuMo with SeSe

C10. Product Distribution RuMo with SeS

Reactants	Products	DG° (kJ/mol)
RuRu + SS	2 RuS	-20.9
RuRu + SeSe	2 RuSe	-27.1
RuRu + SeS	RuS + RuSe	-22.2
MoMo + SS	2 MoS	-21.1
MoMo + SeSe	2 MoSe	-17.3
MoMo + SeS	MoS + MoSe	-17.4
RuMo + SS	RuS + MoS	-15.2
RuMo + SeSe	RuSe + MoSe	-16.4
RuMo + SeS	0.5 (RuS + RuSe + MoS + MoSe)	-14.0

C11. Computed change in Gibbs free energy for all nine reactions

C12. Reaction of RuRu with napEE (1/conc vs time)

C13. Reaction of MoMo with napEE (1/conc vs time)

Supporting information can be found in the link below.

<https://www.dropbox.com/sh/noofmacx05fh3qn/AADGHk0eBluLDxAWv7QFMVNha?dl=0>