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Inhibition of thioredoxin reductase by a novel series of bis-1,2-benzisoselenazol-3(2*H*)-ones: Organoselenium compounds for cancer therapy

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ABSTRACT

Thioredoxin reductase (TrxR) is critical for cellular redox regulation and is involved in tumor proliferation, apoptosis and metastasis. Its C-terminal redox-active center contains a cysteine (Cys497) and a unique selenocysteine (Sec498), which are exposed to solvent and easily accessible. Thus, it is becoming an important target for anticancer drugs. Selective inhibition of TrxR by 1,2-(bis-1,2-benzisoselenazol-3(2*H*)-one)ethane (**4a**) prevents proliferation of several cancer cell lines both in vivo and in vitro. Using the structure of **4a** as a starting point, a series of novel bis-1,2-benzisoselenazol-3(2*H*)-ones was designed, prepared and tested to explore the structure–activity relationships (SARs) for this class of inhibitor and to improve their potency. Notably, 1,2-(5,5′-dimethoxybis(1,2-benzisoselenazol-3(2*H*)-one))ethane (**12**) was found to be more potent than **4a** in both in vitro and in vivo evaluation. Its binding sites were confirmed by biotin-conjugated iodoacetamide assay and a SAR model was generated to guide further structural modification.

1. Introduction

Mammalian thioredoxin reductase (TrxR), together with thioredoxin (Trx) and NADPH, constitutes a critical system for many cellular pathways.[1] Thioredoxins (from mammal to bacteria) are small ubiquitous proteins containing a conserved pair of cysteine residues (–CGPC–) and work as disulfide reductases maintaining cytosolic proteins in their redox state.[1,2] Mammalian TrxRs are large selenoproteins (114 kDa or larger) with conserved C-terminal sequences (–GCUG–) acting as the catalytically active sites.[3,4] The major biological function of TrxR is to reduce oxidized Trx to maintain the cellular antioxidant system.[1,4] It has been reported,[5] however, that the Trx system is involved in many aspects of tumor physiology such as proliferation, apoptosis and metastasis. Researchers[6,7] have also found that the level of TrxR in many tumor cell lines is often more than 10-fold greater than the levels observed in normal cells. Meanwhile, the C-terminal redox-active center of TrxR is protruding away from the enzyme and hence easily accessible for a wide range of substrates.[8] Thus, targeting of the C-terminal active site has become a promising concept for anticancer drug development.[7,9] Many small molecules, such as metal based complexes, curcumin and its analogues, arsenic trioxide and quinines have been reported[10–16] as potent TrxR inhibitors.

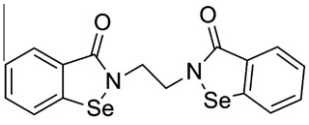

Figure 1. Structure of ethaselen (**4a**).

However, up to the end of 2011, only three anticancer drugs targeting Trx system were under clinical trials: (1) Motexafin gadolinium[17] (MGd), phase III; (2) PX-12[18] (1-methylpropyl 2-imidazolyl disulfide), phase II; (3) Ethaselen (1,2-(bis-1,2-benzisoselenazol-3(2*H*)-one)ethane), also named BBSKE (1,2-[bis(1,2-benzisoselenazolone-3(2*H*)-ketone)]ethane) (**4a**, Fig. 1), phase I. In particular, MGd is a non-competitive inhibitor of rat TrxR (IC_{50} = 6 μM) and induces enzymatic generation of reactive oxygen species (ROS) by TrxR. It also inhibits ribonucleotide reductase (RNR) to exhibit anticancer activity.[19] PX-12 acts as an irreversible inhibitor of cytosolic form thioredoxin (Trx-1) and can also reduce the level of several other plasma proteins.[20] Whereas, ethaselen (**4a**) which is a organoselenium drugs developed by our group, was previously demonstrated[21–24] to be a potent inhibitor of TrxR in rats (IC_{50} = 0.35 μM) that targeting both the Cys497 and Sec498 active

Abbreviations: TrxR, thioredoxin reductase; Trx, thioredoxin; GR, glutathione reductase; SAR, structure–activity relationship; DTNB, 5,5′-dithiol-bis-2-nitrobenzoic acid; LogP_{cal}, calculated Log*P*; BIAM, biotin-conjugated iodoacetamide.

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sites of TrxR and showing remarkable anticancer activity in both in vitro and in vivo experiments. Further reviews[9,25] for this drug are also available. Compared to MGd, ethaselen showed much stronger inhibitory effects on TrxR, whilst they both had no obvious interaction with Trx.[26] In addition, ethaselen can be easily prepared in high yield from readily available starting materials (see Section 4.2). In light of these reports, the novel selenium-containing structure **4a** is considered to have unique biological properties, although structural modification and structure-activity relationships (SARs) study would be necessary to develop promising candidates for future drug discovery.

Herein, using the structure of **4a** as starting point, we describe the design and synthesis of a novel series of bis-1,2-benzisoselenazol-3(2*H*)-ones with the purpose of exploring any SARs. In order to elucidate the relationship between the enzyme inhibitory effects and cell growth inhibition activities of the active compounds and to further confirm their binding sites at the enzyme, several biological evaluations were employed to assess the properties of target compounds. Docking and pharmacological studies of representative compound (**12**) were also carried out.

2. Results and discussion

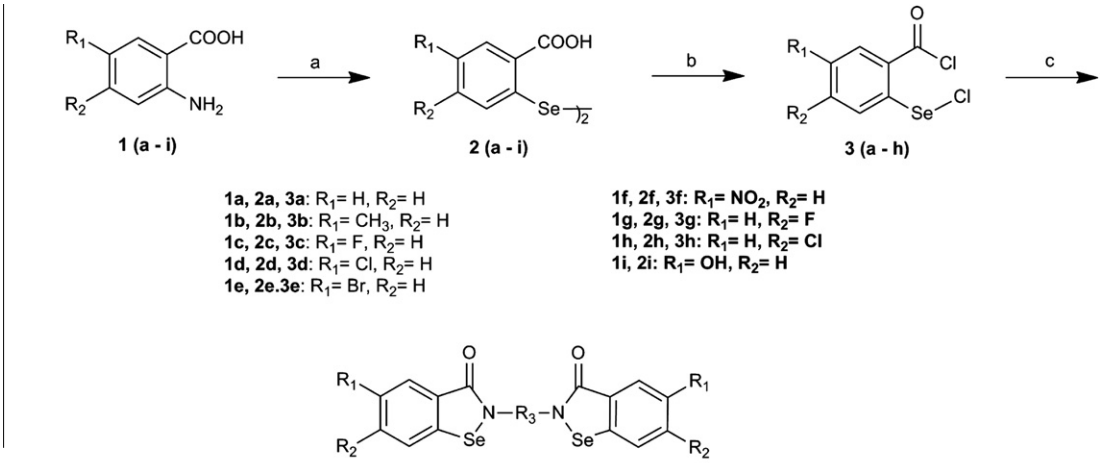
2.1. Chemistry

Although many studies of **4a** concerning its biological and pharmaceutical properties have been published, this is our first time to report the synthesis of **4a** and its analogues. The core bis-1,2-benzisoselenazol-3(2*H*)-one structure was retained and attention was focused predominantly on the introduction of substituents on the aromatic rings, because **4a** was designed to contain two active centers inhibiting both the Cys497 and Sec498 residues of the enzyme. To develop potent analogues of **4a** with favorable biological properties and to explore the SAR of bis-1,2-benzisoselenazol-3(2*H*)-ones as TrxR inhibitors, a series of novel substituted bis-1,2-benzisoselenazol-3(2*H*)-ones was designed and synthesized. With the exception of compounds **12**–**16**, the target compounds were prepared using a simple three-step method (Scheme 1). First, sodium selenide was reacted with the corresponding benzenediazonium salts, themselves prepared from substituted 2-amino benzoic acids, to give 2,2′-diselenobisbenzoic acids. Subsequent heating under reflux with $SOCl_2$ provided the 2-chloroselenobenzoyl chlorides, which were added to the corresponding diamines to give the final products.

For **12** and **13**, the hydroxyl group of compound **2i** was methylated and acetylated, respectively, prior to the chlorination procedure (Scheme 2). Compound **2j** (intermediate of **12**) was prepared by a two-step procedure from **2i**. Briefly, treatment of **2i** with K_2CO_3 and CH_3I afforded dimethyl 5,5′-dimethoxy-2,2′-diselenobisbenzoate, which was used without purification and subsequently hydrolyzed to yield **2j**. Compound **2k** (intermediate of **13**) was synthesized by treating **2i** with acetic anhydride in pyridine, whereas compound **14** was prepared from **13** by hydrolysis of the acetoxy group. Methods for the preparation of **15** and **16** are described in Schemes S1 and S2 (see Supplementary data for details).

2.2. Inhibition of TrxR in vitro

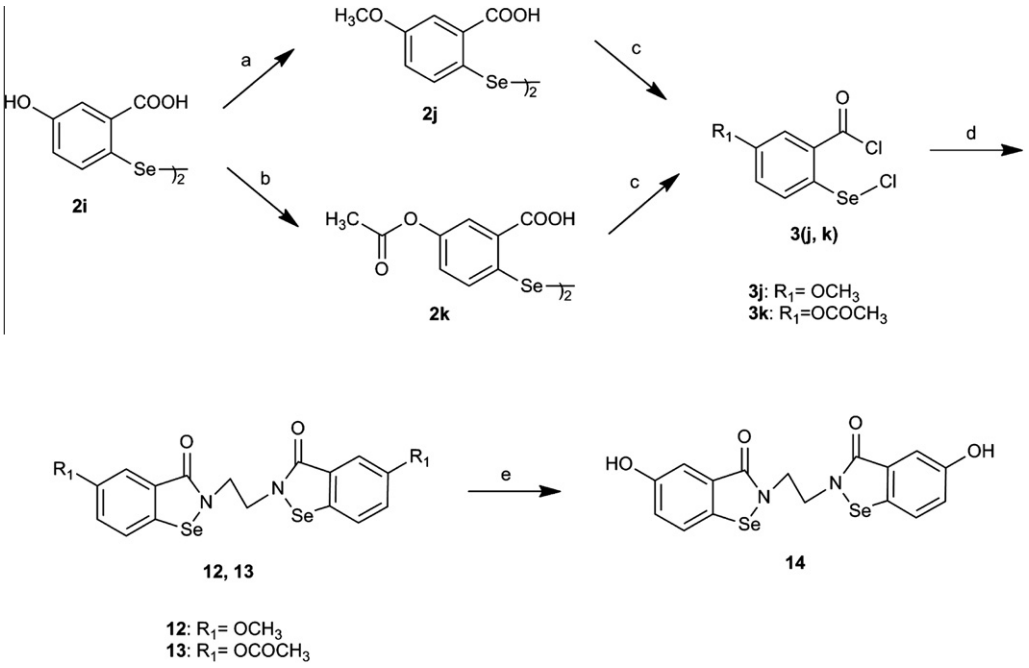
The inhibitory effects of all the target compounds were examined using a 5,5′-dithiol-bis-2-nitrobenzoic acid (DTNB) reduction assay.[8,27] All compounds tested inhibited the DTNB-reducing



4a: R_1= H, R_2= H, R_3= CH_2CH_2
4b: R_1= H, R_2= H, R_3= $CH_2CH_2CH_2$
4c: R_1= H, R_2= H, R_3= $CH_2CH_2CH_2CH_2$
4d: R_1= H, R_2= H, R_3= $CH_2CH_2CH_2CH_2CH_2$
4e: R_1= H, R_2= H, R_3= $CH_2CH_2CH_2CH_2CH_2CH_2$
4f: R_1= H, R_2= H, R_3= $CH_2CH_2CH_2CH_2CH_2CH_2CH_2$
4g: R_1= H, R_2= H, R_3= $CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2$
4h: R_1= H, R_2= H, R_3= cyclohexane
4i: R_1= H, R_2= H, R_3= $PhCH_2$
4j: R_1= H, R_2= H, R_3= Ph
5a: R_1= CH_3, R_2= H, R_3= CH_2CH_2
5b: R_1= CH_3, R_2= H, R_3= $CH_2CH_2CH_2$
5c: R_1= CH_3, R_2= H, R_3= $CH_2CH_2CH_2CH_2$
5d: R_1= CH_3, R_2= H, R_3= $CH_2CH_2CH_2CH_2CH_2$
5e: R_1= CH_3, R_2= H, R_3= $CH_2CH_2CH_2CH_2CH_2CH_2$
5f: R_1= CH_3, R_2= H, R_3= cyclohexane

6a: R_1= F, R_2= H, R_3= CH_2CH_2
6b: R_1= F, R_2= H, R_3= $CH_2CH_2CH_2$
6c: R_1= F, R_2= H, R_3= $CH_2CH_2CH_2CH_2$
6d: R_1= F, R_2= H, R_3= $CH_2CH_2CH_2CH_2CH_2$
6e: R_1= CH_3, R_2= H, R_3= cyclohexane
7a: R_1= Cl, R_2= H, R_3= CH_2CH_2
7b: R_1= Cl, R_2= H, R_3= $CH_2CH_2CH_2CH_2CH_2CH_2$
8a: R_1= Br, R_2= H, R_3= CH_2CH_2
8b: R_1= Br, R_2= H, R_3= $CH_2CH_2CH_2CH_2CH_2CH_2$
9: R_1= NO_2, R_2= H, R_3= CH_2CH_2
10a: R_1= H, R_2= F, R_3= CH_2CH_2
10b: R_1= H, R_2= F, R_3= $CH_2CH_2CH_2CH_2CH_2CH_2$
11a: R_1= H, R_2= Cl, R_3= CH_2CH_2
11b: R_1= H, R_2= Cl, R_3= $CH_2CH_2CH_2CH_2CH_2CH_2$

Scheme 1. Synthesis of compounds **2**–**11**. Reagents: (a) H_2O, $NaNO_2$, HCl, Na_2Se_2; (b) $SOCl_2$, DMF_{cat}; (c) NH_2-R_3-NH_2, triethylamine, CH_3CN or CH_2Cl_2.



Scheme 2. Synthesis of compounds **12**, **13** and **14**. Reagents: (a) DMF, K_2CO_3, CH_3I, then H_2O, NaOH; (b) pyridine, $CH_3COOCOCH_3$, then H_2O; (c) $SOCl_2$, DMF_{cat}; (d) CH_2Cl_2, $NH_2CH_2CH_2NH_2$, triethylamine; (e) DMSO, HCl.

activity of TrxR (TrxR1 of rat liver) in a dose-dependent manner. Their IC_{50} values ranged from 0.1 to more than 50 μM, as shown in Table 1. Several compounds (**4b**, **6a**, **6b**, **9**, **10a**, **12** and **14**) showed good inhibitory effects (IC_{50} <1 μM) which were comparable to or even better (**6a**, **12**, **14**) than that of **4a**. Some SAR information could be derived from these data. It is clear that the enzyme inhibitory activities of the compounds tested were reduced significantly when the selenium atom was replaced by either an oxygen (**15**) or sulfur (**16**) atom. This may result from the Se–N bond being a more electrophilic site for attack from the selenolthiols and thiols of proteins.[28] Furthermore, published research[29] has shown that benzisoselenazolone exhibits a much higher level of reactivity than its benzisothiazolone analogue. In addition, in order to obtain good level of activity, the R_3 group is better to be an alkyl chain because replacement of the alkyl chains (**4a–g**, **5a–e** and **6a–d**) with either cyclohexyl (**4h**, **5f** and **6e**) or aryl (**4i** and **4j**) groups led a significant decrease in the enzyme inhibitory activities. In contrast, among the compounds containing alkyl chains mentioned above, a slight trend of decreased activities can be observed when the number of carbons increased from 2 to more than 5 (except **8a**, **b**). Therefore, the placement of short alkyl chains at R_3 position was preferred for this series of compounds.

Substituents at the R_1 and R_2 positions also influenced the biological activities of the compounds tested. Compound **12** (methoxyl in R_1) showed the best inhibitory effect (IC_{50} = 0.13 μM), whereas compounds **6a** (fluoro in R_1) and **14** (hydroxyl in R_1) were less active (IC_{50} = 0.28 μM and 0.32 μM, respectively) but still better than **4a**. Among compounds with halogen atom substituents, fluoro containing ones (e.g. **6a** and **10a**) were more active than their chloro and bromo analogues (e.g. **7a**, **8a** and **11a**), although they all showed good inhibitory activities (IC_{50} <3 μM).

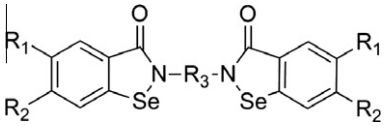
2.3. Growth inhibition of cancer cells

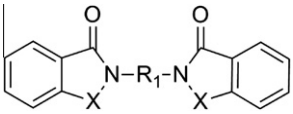
In order to identify novel anticancer agents, the inhibitory effects of selected compounds on the growth of human cancer cell lines, including U87 MG (glioma), MIA PaCa-2 (pancreatic carcinoma) and LoVo (colon cancer), were examined. The results are summarized in Table 2. As expected, compounds with good inhibitory activities of TrxR in vitro (IC_{50} <1 μM), such as **6a**, **12** and **14**, generally showed comparable activities of cell growth inhibition with regard to **4a**. This suggests a possible link between TrxR inhibition and cell growth inhibition of the tested compounds (see Section 2.4). For compound **12**, improvement of growth inhibition activity in all tested cell lines can be observed in comparison to **4a**. However, compounds containing chloro or bromo substituents (**7a**, **8a** and **11a**) exhibited very poor inhibitory effects on cancer cell lines (IC_{50} >50 μM), whereas their fluoro analogue (**6a** and 1**0a**) were much more active (IC_{50} between 3 and 13 μM in all tested cell lines, Table 2). Subsequent calculation of the Log*P* values for all of the compounds, revealed that compounds with halogen atoms (except for fluoro) showed higher LogP_{cal} with the LogP_{cal} values of **7a**, **8a** and **11a** being 4.45, 5.71 and 4.49, respectively. In contrast, the LogP_{cal} of all the other compounds (tested in cell growth inhibition) were less than 4. Precipitation of small amounts of compounds (**7a**, **8a** and **11a**) was also observed when they were incubated with cells in medium for 48 h during the MTT assay. It was postulated that the poor inhibitory activities on cell growth of the chloro or bromo containing compounds were caused by their high lipophilicity (large Log*P*) and high molecular weight and that these properties made them less active in vivo than in vitro because limited solubility and large molecular weight hindered their cellular permeability.

2.4. Inhibition of cellular TrxR

To substantiate the assumption made above and to clarify the possible link between TrxR inhibition and cell growth inhibition, U87 MG cell line was selected to investigate the inhibitory effects of five representative compounds (**12**, **4a**, **5a**, **8a** and **9**) on the activity of cellular TrxR. Exposure of U87 MG cells to 1–20 μM solutions of different compounds showed that cellular TrxR activity was attenuated to varying degrees (Fig. 2A). Encouragingly, compound **12** showed great inhibitory effect on cellular TrxR with an IC_{50} value of approximately 2 μM, which was better than the effect of **4a** (IC_{50} ~5 μM). TrxR activity in U87 MG cell extracts was inhibited by more than 90% when the concentration of **12** reached 20 μM. In fact, compounds **4a**, **5a**, **8a** and **9** exhibited dose-depen-

Table 1
In vitro TrxR inhibitory activities of the novel series of bis-1,2-benzisoselenazol-3(2*H*)-ones[a]



Compound	R_1	R_2	R_3	IC_{50} (μM)
4a	H	H	CH_2CH_2	0.35[b]
4b	H	H	$CH_2CH_2CH_2$	0.78 ± 0.03
4c	H	H	$CH_2CH_2CH_2CH_2$	1.03 ± 0.05
4d	H	H	$CH_2CH_2CH_2CH_2CH_2$	1.95 ± 0.15
4e	H	H	$CH_2CH_2CH_2CH_2CH_2CH_2$	1.66 ± 0.29
4f	H	H	$CH_2CH_2CH_2CH_2CH_2CH_2CH_2$	5.85 ± 0.57
4g	H	H	$CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2$	4.49 ± 0.76
4h	H	H	Cyclohexane	12.79 ± 1.21
4i	H	H	$PhCH_2$	47.68 ± 10.28
4j	H	H	Ph	34.30 ± 7.45
5a	CH_3	H	CH_2CH_2	1.26 ± 0.31
5b	CH_3	H	$CH_2CH_2CH_2$	1.16 ± 0.21
5c	CH_3	H	$CH_2CH_2CH_2CH_2$	2.38 ± 0.03
5d	CH_3	H	$CH_2CH_2CH_2CH_2CH_2$	3.16 ± 0.05
5e	CH_3	H	$CH_2CH_2CH_2CH_2CH_2CH_2$	4.91 ± 0.31
5f	CH_3	H	Cyclohexane	>50
6a	F	H	CH_2CH_2	0.28 ± 0.04
6b	F	H	$CH_2CH_2CH_2$	0.86 ± 0.03
6c	F	H	$CH_2CH_2CH_2CH_2$	1.64 ± 0.19
6d	F	H	$CH_2CH_2CH_2CH_2CH_2$	3.87 ± 1.02
6e	F	H	Cyclohexane	10.49 ± 2.14
7a	Cl	H	CH_2CH_2	1.31 ± 0.11
7b	Cl	H	$CH_2CH_2CH_2CH_2CH_2$	1.80 ± 0.74
8a	Br	H	CH_2CH_2	1.58 ± 0.52
8b	Br	H	$CH_2CH_2CH_2CH_2CH_2$	1.59 ± 0.29
9	NO_2	H	CH_2CH_2	0.57 ± 0.01
10a	H	F	CH_2CH_2	0.82 ± 0.11
10b	H	F	$CH_2CH_2CH_2CH_2CH_2$	2.91 ± 0.80
11a	H	Cl	CH_2CH_2	2.87 ± 0.51
11b	H	Cl	$CH_2CH_2CH_2CH_2CH_2$	4.54 ± 0.91
12	OCH_3	H	CH_2CH_2	0.13 ± 0.02
13	$OCOCH_3$	H	CH_2CH_2	22.60 ± 4.05
14	OH	H	CH_2CH_2	0.32 ± 0.02
				
15	O		CH_2CH_2	>100
16	S		CH_2CH_2	48.09 ± 1.91

[a] Values are means and SD of at least two experiments.
[b] Cite from our previous study (see Ref. 22).

Table 2
Growth inhibition activities of selected compounds[a].

Compound	IC_{50} (μM) of cell lines		
	Mia-paca2	LoVo	U87
4a	3.79 ± 0.19	7.67 ± 0.23	6.41 ± 0.30
4d	3.27 ± 0.20	5.50 ± 0.96	6.05 ± 0.67
4h	11.50 ± 0.74	17.22 ± 0.48	>50
4i	>50	>50	ND
4j	>50	ND	>50
5a	5.94 ± 0.89	6.12 ± 0.63	17.77 ± 0.09
5d	6.71 ± 0.92	16.56 ± 0.21	12.25 ± 2.39
6a	3.07 ± 0.45	4.55 ± 0.13	6.95 ± 0.41
7a	>50	ND[b]	>50
8a	>50	ND	>50
9	>50	ND	>50
10a	8.47 ± 0.57	ND	12.60 ± 1.25
11a	>50	ND	>50
12	2.84 ± 0.30	5.28 ± 0.54	1.64 ± 0.25
13	10.23 ± 1.23	ND	20.76 ± 2.78
14	5.30 ± 0.10	6.53 ± 1.77	6.60 ± 1.11

[a] All values are shown in IC_{50} (μM) values and expressed as means ± SD of triplicate experiments.
[b] ND = No data.

dent inhibition of the target. Interestingly, comparison of these results with the cell growth inhibition activities of the selected compounds (Fig. 2B) revealed good correlation (Table 3) and demonstrated that compounds with higher cell growth inhibition activities ultimately displayed better percentile inhibitions of intracellular TrxR and vice versa. This result, combined with observations from our previous study[21] of **4a**, indicated that the cell growth inhibitory activities of this series of bis-1,2-benzisoselenazol-3(2*H*)-ones could be attributed to their inhibitory effects of intracellular TrxR. Again, **8a** showed weak inhibitory activity consolidating our assumptions, whereas compound **9**, which was a potent TrxR inhibitor in vitro (IC_{50} = 0.57 μM), exhibited very poor activity in both cell growth inhibition and TrxR inhibition in vivo, in spite of a low Log*P* ($Log P_{cal}$ = 2.98) and low molecular weight in comparison to **8a**. The reason for this abnormal property of **9** would require further study.

2.5. Detection of binding sites

According to our initial design, it was assumed that compound **12** would interact with the enzyme at both the Sec498 and Cys497

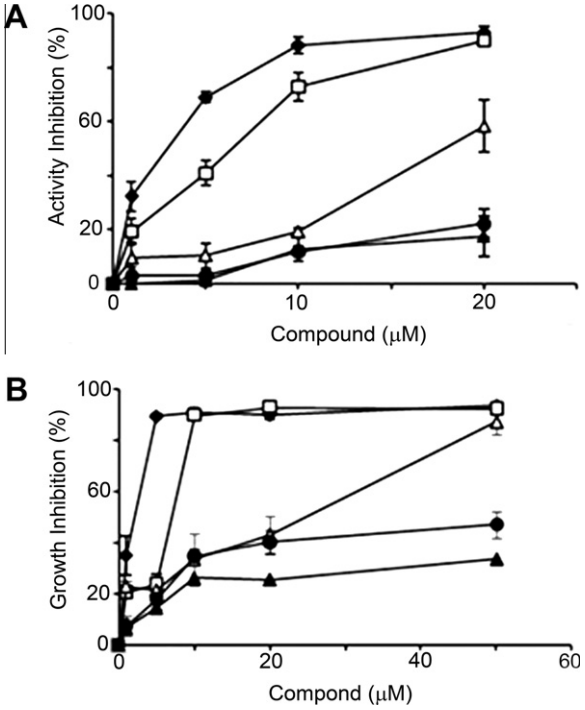


Figure 2. Comparison between cellular TrxR inhibitory effects and cell growth inhibitory activities of selected compounds (**12**, **4a**, **5a**, **8a** and **9**). (A) Dose-dependent curves of the inhibitory effects of tested compounds of cellular TrxR in U87 MG cell lines. (B) Dose-dependent curves of U87 MG cell growth inhibitory activities of tested compounds. Symbols were used for **12** (♦), **4a** (□), **5a** (△), **8a** (●) and **9** (▲). See Section 2.4 for discussion.

Table 3
The correlation coefficient between cell growth inhibition and TrxR inhibition of the compounds tested

Compounds	Correlation coefficient (r)[a]
12	0.98
4a	0.96
5a	0.85
8a	0.92
9	0.89

[a] The values of r were calculated and represented by pearson product-moment correlation coefficient.

residues of the C-terminal active sites of TrxR. In order to confirm its binding sites, a biotin-conjugated iodoacetamide (BIAM) assay was conducted. It was reported[30] that BIAM can react with TrxR selectively by adjusting the pH value. At a pH of 6.5 only the Sec498 residue was alkylated by BIAM, whereas at a pH of 8.5, both the Sec498 and Cys497 residues were alkylated by BIAM. In the current study, the blotting result confirmed that **12** can react with both sites of the C-terminal redox-active center because the bands of the experimental groups were much weaker than the control groups at both pH 8.5 and 6.5 (Fig. 3). Moreover, **12** blocked both active sites of TrxR at either pH (8.5 or 6.5) in a dose-dependent manner. Under physiological conditions, the pK_a value of the –SeH group in Sec is 6.5[31] whereas that of the –SH in Cys is 8.5.[32] It is clear that the selenocysteine is more reactive than cysteine under physiological conditions because the former is present as the predominant form of selenide. Therefore, it was speculated that **12** inhibits TrxR in a two-step mechanism which is similar to that of other reported TrxR inhibitors.[8,33] First, the ionized –SeH group of Sec498 acts as a nucleophilic agent and attack the selenium atom of a single isoselenazolone ring in **12** because the Se–N bond of isoselenazolones reacts in a facile manner with selenothiols and

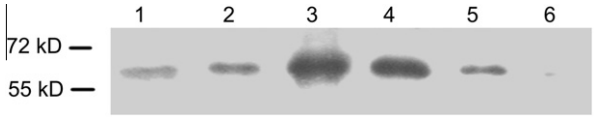


Figure 3. Binding sites detection of **12** at TrxR. Different concentrations of **12** were added to reduced TrxR (1.1 μM) and incubated at room temperature for 2 h. The same amounts of DMSO were added to the control experiments. *Lanes* 1–3, pH 8.5; *Lanes* 4–6, pH 6.5; *Lanes* 2, 5, 10 μM; *Lanes* 1, 6, 20 μM; *Lanes* 3, 4, control.

thiols.[28] Subsequent reaction of the other isoselenazolone ring with the neighboring –SH group of Cys497 leads to a double-blocking of the redox-active site of TrxR. This hypothesis has been supported either by glutathione reductase (GR) inhibition assays in which only minor inhibition was observed for the compounds tested (see Supplementary data, Fig. S1) or by our previous study[26] in which **4a** exhibited poor or no inhibitory effects of Sec498 mutant (Sec to Cys) or C-terminal truncated human TrxR.

2.6. Docking study

To better understand the potency of compound **12** and to guide further SAR study, we carried out docking study of it with crystallographic structure of TrxR. There was evidence[34] showing that small molecules could bind to TrxR in the cavity between the C-terminal and N-terminal active sites. For the current docking study, the crystallographic structure of human thioredoxin reductase I (PDB code: 2J3N) was selected and the conformation of the enzyme used for the experiment was presumed to be the favored and solvent stable form.[34] A docking sphere in the cavity between the two redox-active centers (Cys59, Cys 64 at the N-terminal and Cys497, Sec498 at the C-terminal) was created. As illustrated in Figure 4, following the hydrogen bond formation between one of its carbonyl group and hydrogen of His472, one unit of benzisoselenazolone heterocycle of **12** inserted into the presumed 'pocket' consisting of His108, Trp407, Pro408 and Leu409 while another unit of benzisoselenazolone formed a hydrogen bond with Lys29. Furthermore, hydrogen bonds between the two methoxy groups of **12** and Lys123 and Trp407 were also observed. It was demonstrated[34,36,37] that His472, Tyr116, Trp407 and Lys29 played a very important role for catalysis of TrxR by means of facilitating its reduction. This result, along with biological assays date, suggests that compound **12** is a potential inhibitor of TrxR.

2.7. Pharmacological properties of 12

Pharmacological studies revealed that **12** inhibited TrxR in a rapid, mixed-type and reversible mechanism (Fig. 5). Michaelis–Menten analysis (Fig. 5A) and Lineweaver–Burk plot (Fig. 5B) suggested that **12** acts as a mixed-type inhibitor of TrxR with regard to DTNB, as opposed to a pure competitive inhibitor. This can be explained by the proposed double-blocking inhibition in which both Cys497 and Sec498 are modified covalently by **12**. When the concentration of inhibitor increases, the Michaelis–Menten Constant (K_m) increases (the increasing x-intercept in Fig. 5B) as a higher concentration of DTNB is required to reach the K_m, because the inhibitor and DTNB react with the same active sits of TrxR. This type of inhibition, however, cannot be overcome by high concentrations of DTNB as covalent bonds are formed, so the Maximal Rate of Reaction (V_{max}) decreases (increasing y-intercept in Fig. 5B). Furthermore, **12** inhibits TrxR in a rapid but time-dependent manner (Fig. 5C). However, the overall reaction is largely reversible by ultrafiltration assay (Fig. 5D). This may due to the recyclization of the benzisoselenazolone ring or cleavage of the Se–Se bond between the selenium atoms of the inhibitor and the Sec498 residue by the sulfur atom of Cys497, before an

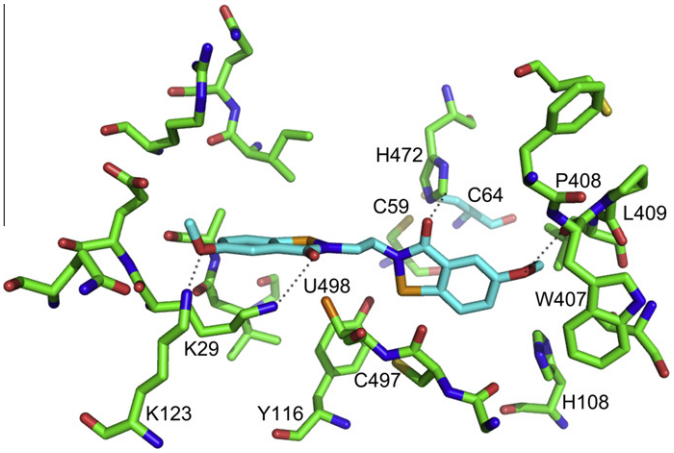


Figure 4. Representation of the putative binding modes of compound **12** (cyan carbon) in the 'pocket' between the two redox-active centers of TrxR. The ligand and Residues closed to it are displayed as sticks. Hydrogens are hidden for clarity. Standard atom color codes: carbons in residues, green; oxygen, pink; nitrogen, blue; sulfur, yellow; selenium, orange. Hydrogen bonds between ligands and residues are symbolized by grey dash lines.

intermolecular bond between Cys497 and another selenium atom of the inhibitor can be formed. As a result, therefore, the inhibitor can be released from the active sites.

3. Conclusion

In Figure 6, the SARs for bis-1,2-benzisoselenazol-3(2*H*)-ones as TrxR inhibitors is summarized based on the biological assays and

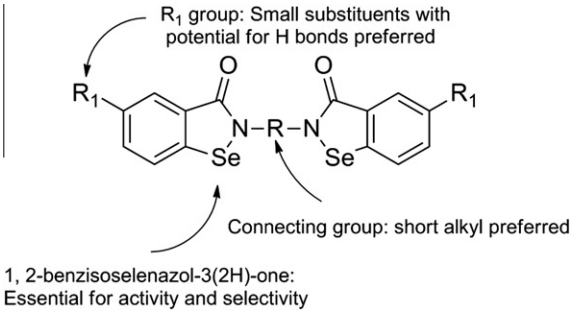


Figure 6. SAR summary for bis-1,2-benzisoselenazol-3(2*H*)-ones as TrxR inhibitors.

docking studies described above. Briefly, selenium is essential to the biological activity of this series of compounds and the linker between the two benzisoselenazolone heterocycles should be a short alkyl group. Furthermore, small substituents on the benzene ring with the potential for hydrogen bond formation are preferred in order to reduce the molecular lipophilicity. As TrxR receives increasing attention as drug target, especially for anticancer therapy, these SARs and docking studies may provide models for the future development of organoselenium compounds as TrxR inhibitors. Encouragingly, the discovery of **12** substantiates the assertion that structural modification of **4a** was necessary and feasible to improve the biological activity of this series of compounds. This study also confirms that both Cys497 and Sec498 are binding targets for **12**. In addition, the enzyme inhibitory effects of the compounds tested demonstrates the close relationship with their anticancer activities according to our comparison between the

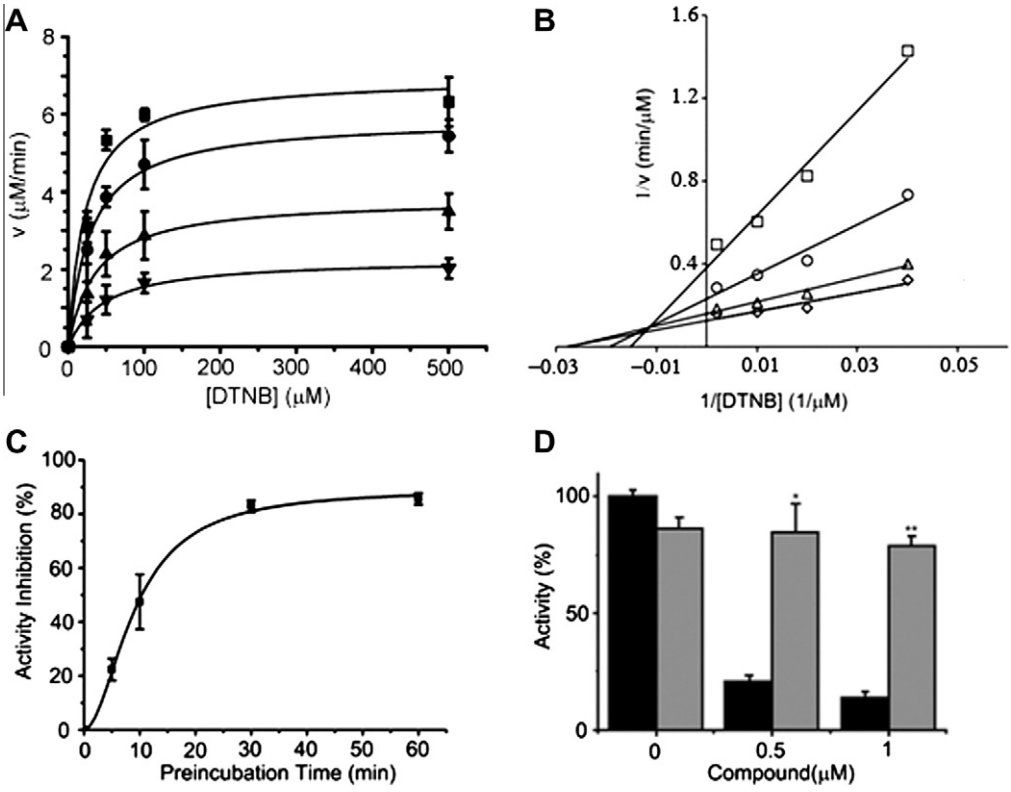


Figure 5. Characterization of TrxR inhibition by **12** (A) Michaelis–Menten analysis of DTNB reduction assay, control (no inhibitor, ■); 50 nM (●); 200 nM (▲) and 500 nM (▼); concentrations of DTNB were set as 25, 50, 100 and 500 μM. (B) Mixd-type inhibition of TrxR was determined by Lineweaver-Burk plots, control (no inhibitor, ◇); 50 nM (△); 200 nM (○) and 500 nM (□). (C) TrxR is inhibited in a time-dependent manner; concentrations of DTNB were set as 25, 50, 100 and 500 μM. (D) Reversibility of TrxR inhibition was tested by ultrafiltration assay, black, before ultrafiltration; grey, after ultrafiltration. $^{*}P$ <0.005 vs before ultrafiltration (0.5 μM); $^{**}P$ <0.001 vs before ultrafiltration (1 μM). Data was obtained by triplicate experiments and presented as mean ± SD.

results of MTT assays and cellular TrxR inhibition assays. Although the exact molecular mechanism of TrxR inhibition by these compounds requires further clarification, given the complex kinetic behavior exhibited by **12** during its reaction with the enzyme, the development of this novel series of bis-1,2-benzisoselenazol-3(2*H*)-ones offers a solid basis for designing potent organoselenium compounds as anticancer drugs by targeting TrxR.

4. Experimental

4.1. Chemicals and methods

All chemical reagents were commercially available (purchased from Sigma–Aldrich, Alfa Aesar and TCI in high quality) and were used without further purification unless otherwise stated. $SOCl_2$ was distilled prior to use. ^1H and ^{13}C NMR spectra were obtained on a Bruker Avance III instrument, 400 MHz for ^1H spectra and 100 MHz for ^{13}C spectra, in DMSO-d_6 or $CDCl_3$. Mass spectra were run on an APEX IV FT-MS (7.0T) instrument. The purity of all compounds tested was determined by HPLC analysis (Agilent 1100, Agilent Technologies, Inc., USA) with a flow rate of 1 ml/min at 25 °C (Ultimate XB-C18 column, 5 μm, 4.6 × 250 nm, Welch Materials, Inc.). The samples were eluted with Milli-Q water containing 0.1% phosphate and methanol, using UV monitor at 254 nm for detection. Retention times (t_R) were calculated in minutes, and purity was calculated as % total area.

4.2. Synthesis

4.2.1. General procedure for the synthesis of 2(a–i)

2-Amino benzoic acid (0.1 mol), HCl (20 ml) and H_2O (20 ml) were added to a beaker and the mixture was cooled at 0 °C. An aqueous solution of sodium nitrite (0.11 mol) (30%) was added slowly added and the reaction mixture was stirred for 30 min. The mixture was then poured into an aqueous solution of disodium diselenide (0.05 mol) and the resulting solution was stirred at room temperature for another 3 h before the pH was adjusted with aqueous HCl (10%) to a value of 2–3. Precipitation occurred and the final products were obtained by filtration.

4.2.1.1. 2,2′-diselenobisbenzoic acid (2a). Compound **1a** (13.7 g, 0.1 mol) was used as reactant to give 19.6 g of **2a** as yellow solid (95%). ^1H NMR (400 MHz, DMSO-d_6) δ 13.67 (br, 2H, COOH), 8.00 (d, J = 7.6 Hz, 2H, ArH), 7.66 (d, J = 7.6 Hz, 2H, ArH), 7.45 (t, J = 7.6 Hz, 2H, ArH), 7.33 (t, J = 7.6 Hz, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.62, 133.43, 133.15, 131.41, 129.65, 129.43, 126.32. MS (EI) m/z: 401.9 (M^+).

4.2.1.2. 5,5′-dimethyl-2,2′-diselenobisbenzoic acid (2b). Compound **1b** (15.1 g, 0.1 mol) was used as reactant to give 20.3 g of **2b** as yellow solid (90%). ^1H NMR (400 MHz, DMSO-d_6) δ 13.55 (br, 2H, COOH), 7.84 (d, J = 1.4 Hz, 2H, ArH), 7.50 (d, J=8.2 Hz, 2H, ArH), 7.29 (dd, J_1 = 8.2 Hz, J_2=1.4 Hz, 2H, ArH), 2.29 (s, 6H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.52, 135.94, 134.33, 131.82, 129.84, 129.45, 128.52, 20.07. MS (EI) m/z: 429.9 (M^+).

4.2.1.3. 5,5′-difluoro-2,2′-diselenobisbenzoic acid (2c). Compound **1c** (15.5 g, 0.1 mol) was used as reactant to give 19.9 g of **2c** as yellow solid (91%). ^1H NMR (400 MHz, DMSO-d_6) δ 14.07 (br, 2H, COOH), 7.78 (dd, J_1 = 9.1, J_2 = 2.8, 2H, ArH), 7.65 (dd, J_1 = 8.5, J_2 = 5.2, 2H, ArH), 7.42 (td, J_1 = 8.5, J_2 = 2.8, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.54, 160.91 (d, J_{C-F} = 244.5), 131.65 (d, J_{C-F} = 7.2), 130.34 (d, J_{C-F} = 6.7), 128.18 (d, J_{C-F} = 2.5), 120.96 (d, J_{C-F} = 21.6), 117.85 (d, J_{C-F} = 23.0). MS (EI) m/z: 437.9 (M^+).

4.2.1.4. 5,5′-dichloro-2,2′-diselenobisbenzoic acid (2d). Compound **1d** (17.1 g, 0.1 mol) was used as reactant to give 20.0 g of **2d** as yellow solid (85%). ^1H NMR (400 MHz, DMSO-d_6) δ 14.12 (br, 2H, COOH), 8.00 (d, J = 2.2, 2H, ArH), 7.64 (d, J = 8.6, 2H, ArH), 7.60 (dd, J_1 = 8.6, J_2 = 2.2, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.92, 144.59, 133.82, 132.55, 132.04, 131.26, 130.89. MS (EI) m/z: 469.8 (M^+).

4.2.1.5. 5,5′-dibromo-2,2′-diselenobisbenzoic acid (2e). Compound **1e** (21.5 g, 0.1 mol) was used as reactant to give 21.5 g of **2e** as yellow solid (77%). ^1H NMR (400 MHz, DMSO-d_6) δ 14.06 (br, 2H, COOH), 8.10 (d, J = 2.3, 2H, ArH), 7.69 (dd, J_1 = 8.6, J_2 = 2.3, 2H, ArH), 7.55 (d, J = 8.6, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.55, 145.11, 138.92, 136.65, 130.36, 128.77, 120.31. MS (EI) m/z: 557.7 (M^+).

4.2.1.6. 5,5′-dinitro-2,2′-diselenobisbenzoic acid (2f). Compound **1f** (18.2 g, 0.1 mol) was used as reactant to give 17.0 g of **2f** as brown solid (69%). ^1H NMR (400 MHz, DMSO-d_6) δ 13.40 (br, 2H, COOH), 8.58 (d, J = 2.3, 2H, ArH), 8.07 (dd, J_1 = 8.8, J_2 = 2.3, 2H, ArH), 7.91 (d, J = 8.8, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.90, 151.5, 149.60, 132.03, 130.95, 128.76, 125.42. MS (EI) m/z: 461.9 (M^+).

4.2.1.7. 4,4′-difluoro-2,2′-diselenobisbenzoic acid (2g). Compound **1g** (15.5 g, 0.1 mol) was used as reactant to give 17.1 g of **2g** as yellow solid (78%). ^1H NMR (400 MHz, DMSO-d_6) δ 13.92 (br, 2H, COOH), 8.12 (dd, J_1 = 8.4, J_2 = 6.0, 2H, ArH), 7.40 (dd, J_1 = 9.8, J_2 = 2.3, 2H, ArH), 7.23 (td, J_1 = 8.4, J_2 = 2.3, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.76, 162.52 (d, J_{C-F} = 250.5), 136.64 (d, J_{C-F} = 8.5), 134.46 (d, J_{C-F} = 20.8), 125.77 (d, J_{C-F} = 9.3), 116.09 (d, J_{C-F} = 3.5), 114.05 (d, J_{C-F} = 25.0). MS (EI) m/z: 438.0 (M^+).

4.2.1.8. 4,4′-dichloro-2,2′-diselenobisbenzoic acid (2h). Compound **1h** (17.1 g, 0.1 mol) was used as reactant to give 20.7 g of **2h** as yellow solid (88%). ^1H NMR (400 MHz, DMSO-d_6) δ 14.08 (br, 2H, COOH), 8.03 (d, J = 8.3, 2H, ArH), 7.60 (d, J = 1.7, 2H, ArH), 7.44 (dd, J_1 = 8.3, J_2 = 1.7, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.56, 150.02, 137.26, 137.07, 133.18, 129.34, 125.58. MS (EI) m/z: 469.9 (M^+).

4.2.1.9. 5,5′-dihydroxy-2,2′-diselenobisbenzoic acid (2i). Compound **1i** (15.3 g, 0.1 mol) was used as reactant to give 14.5 g of **2i** as brown solid (67%). ^1H NMR (400 MHz, DMSO-d_6) δ 13.16 (br, 2H, COOH), 9.82 (s, 2H, OH), 7.44 (d, J = 2.8, 2H, ArH), 7.42 (d, J = 8.7, 2H, ArH), 6.93 (dd, J_1 = 8.7, J_2 = 2.8, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.39, 156.24, 130.75, 129.28, 121.34, 121.18, 117.71. MS (EI) m/z: 433.9 (M^+).

4.2.1.10. 5,5′-dimethoxy-2,2′-diselenobisbenzoic acid (2j). The preparation of **2j** was a two-step process. Step 1: **2i** (4.3 g, 0.01 mol) was dissolved in 100 ml DMF containing 6.5 g K_2CO_3, to which 7.2 ml CH_3I was added dropwise under stirring and the mixture was stirred for 14 h at 50 °C. Then the mixture was poured into 200 ml water and this solution was extracted by diethyl ether (5 × 50 ml) and washed by 0.1 M NaOH (3 × 20 ml) and water (50 ml). The ether extract was dried by Na_2SO_4 followed by the removal of solvent under vacuum to give 3 g yellow powder without further purification. Step 2: the yellow powder was dissolved in 50 ml methanol and 80 ml water containing 2 g NaOH. The reaction mixture was stirred under reflux and the solution became transparent after 1 h. The solution was stirred for another 1 h. After that, HCl was added to adjust the pH to 2. The mixture was filtrated to give 2.3 g of **2j**. Yellow solid (50%). ^1H NMR (400 MHz, DMSO-d_6) δ 12.13 (br, 2H, COOH), 7.60 (d, J = 8.8, 2H,

ArH), 7.20 (d, J = 2.7, 2H, ArH), 7.13 (dd, J_1 = 8.8, J_2 = 2.7, 2H, ArH), 3.75 (s, 6H, OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.20, 158.18, 130.75, 129.64, 123.26, 120.22, 115.96, 55.52. MS (ESI) m/z: 460.9 (M^+-H).

4.2.1.11. 5,5′-diacetoxy -2,2′-diselenobisbenzoic acid (2k). Compound **2i** (4.3 g, 0.01 mol), 40 ml acetic anhydride and 3 ml pyridine were used as reactants and the mixture was under reflux at 100 °C for half an hour. Then it was poured into 100 ml water and was stirred for another 1 h, after which it was extracted by ethyl acetate and the organic solvent was dried and removed to give 3.0 g of **2k**. White solid (59%). ^1H NMR (400 MHz, DMSO-d_6) δ 13.98 (br, 2H, COOH), 7.78 (d, J = 2.6, 2H, ArH), 7.67 (d, J = 8.7, 2H, ArH), 7.31 (dd, J_1 = 8.7, J_2 = 2.6, 2H, ArH), 2.25 (s, 6H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.13, 167.81, 151.37, 149.17, 130.67, 130.02, 129.50, 127.32, 20.77. MS (EI) m/z: 518.0 (M^+).

4.2.2. General procedure for the synthesis of 3(a–k)

2,2′-Diselenobisbenzoic acid (0.01 mol) was added to thionyl chloride (20 ml) and DMF (0.5 ml) was added as a catalyst. The reaction mixture was stirred at reflux (85 °C) for 3 h. The solvents were evaporated under vacuum and the crude products were purified by recrystallization from cyclohexane.

4.2.2.1. 2-(Chloroseleno)benzoyl chloride (3a). Compound **2a** (4 g, 0.01 mol) was used as reactant to give 4.1 g of **3a** as orange crystal (81%). ^1H NMR (400 MHz, $CDCl_3$) δ 8.09 (d, J = 7.7, 1H, ArH), 7.85 (d, J = 7.7, 1H, ArH), 7.67 (t, J = 7.7, 1H, ArH), 7.39 (t, J = 7.7, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.51, 136.19, 134.54, 131.29, 129.03, 127.41, 126.42.

4.2.2.2. 5-Methyl-2-(chloroseleno)benzoyl chloride (3b). Compound **2b** (4.3 g, 0.01 mol) was used as reactant to give 4.7 g of **3b** as orange crystal (87%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.96 (d, J = 8.2, 1H, ArH), 7.65 (s, 1H, ArH), 7.48 (d, J = 8.2, 1H, ArH), 2.37 (s, 6H, CH3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.74, 141.63, 135.72, 134.17, 129.61, 127.44, 126.38, 20.66.

4.2.2.3. 5-Fluoro-2-(chloroseleno)benzoyl chloride (3c). Compound **2c** (4.4 g, 0.01 mol) was used as reactant to give 4.1 g of **3c** as orange crystal (76%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.78 (dd, J_1 = 9.0, J_2 = 2.7, 1H, ArH), 7.65 (dd, J_1 = 8.3, $J2$ = 5.4, 1H, ArH), 7.42 (td, J_1 = 8.3, J_2 = 2.7, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.84, 161.41 (d, J_{C-F} = 248.6), 131.65 (d, J_{C-F} = 7.5), 130.34 (d, J_{C-F} = 8.7), 128.18 (d, J_{C-F} = 2.2), 120.96 (d, J_{C-F} = 24.3), 117.85 (d, J_{C-F} = 22.5).

4.2.2.4. 5-Chloro-2-(chloroseleno)benzoyl chloride (3d). Compound **2d** (4.7 g, 0.01 mol) was used as reactant to give 4.4 g of **3d** as orange crystal (76%). ^1H NMR (400 MHz, $CDCl_3$) δ 8.12 (d, J = 8.6, 1H, ArH), 7.69 (d, J = 2.2, 1H, ArH), 7.65 (dd, J_1 = 8.6, J_2 = 2.2, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.70, 144.40, 136.12, 133.58, 133.06, 130.02, 128.51.

4.2.2.5. 5-Bromo-2-(chloroseleno)benzoyl chloride (3e). Compound **2e** (5.6 g, 0.01 mol) was used as reactant to give 4.7 g of **3e** as yellow crystal (71%). ^1H NMR (400 MHz, $CDCl_3$) δ 8.14 (d, J = 2.2, 1H, ArH), 7.65 (dd, J_1 = 8.6, J_2 = 2.2, 1H, ArH), 7.49 (d, J = 8.6, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.55, 145.11, 138.90, 136.60, 130.34, 128.70, 120.33.

4.2.2.6. 5-Nitro-2-(chloroseleno)benzoyl chloride (3f). Compound **2f** (4.9 g, 0.01 mol) was used as reactant to give 4.0 g of **3f** as yellow crystal (67%). ^1H NMR (400 MHz, $CDCl_3$) δ 8.32 (d, J = 2.1, 1H, ArH), 8.01 (dd, J_1 = 8.5, J_2 = 2.1, 1H, ArH), 7.89 (d, J = 8.5, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.05, 153.96, 146.17, 129.59, 127.93, 126.01, 122.91.

4.2.2.7. 4-Fluoro-2-(chloroseleno)benzoyl chloride (3g). Compound **2g** (4.4 g, 0.01 mol) was used as reactant to give 4.5 g of **3g** as orange crystal (83%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.86 (dd, J_1 = 9.9, J_2 = 2.3, 1H, ArH), 7.80 (dd, J_1 = 8.4, J_2 = 5.7, 1H, ArH), 7.19 (td, J_1 = 8.4, J_2 = 2.3, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.83, 160.44, 147.83, 144.46, 135.26, 127.44, 127.10.

4.2.2.8. 4-Chloro-2-(chloroseleno)benzoyl chloride (3h). Compound **2h** (4.7 g, 0.01 mol) was used as reactant to give 5.0 g of **3h** as orange crystal (87%). ^1H NMR (400 MHz, $CDCl_3$) δ 8.13 (d, J = 1.8, 1H, ArH), 7.74 (d, J = 8.1, 1H, ArH), 7.40 (dd, J_1 = 8.1, J_2 = 1.8, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.61, 151.20, 136.24, 135.47, 132.15, 128.54, 124.58.

4.2.2.9. 5-Methoxy-2-(chloroseleno)benzoyl chloride (3j). Compound **2j** (4.6 g, 0.01 mol) was used as reactant to give 3.9 g of **3j** as orange crystal (68%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.90 (d, J = 8.8, 1H, ArH), 7.39 (d, J = 2.7, 1H, ArH), 7.33 (dd, J_1 = 8.8, J_2 = 2.7, 1H, ArH), 3.80 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.53, 158.31, 134.70, 127.28, 127.22, 121.64, 112.50, 55.64.

4.2.2.10. 5-Acetoxy-2-(chloroseleno)benzoyl chloride (3k). Compound **2k** (5.2 g, 0.01 mol) was used as reactant to give 4.1 g of **3k** as orange crystal (65%). ^1H NMR (400 MHz, $CDCl_3$) δ 8.13 (d, J = 2.4, 1H, ArH), 8.10 (d, J = 8.8, 1H, ArH), 7.57 (dd, J_1 = 8.8, J_2 = 2.4, 1H, ArH), 2.40 (s, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.02, 169.05, 149.47, 143.04, 130.23, 129.60, 128.29, 126.90, 21.11.

4.2.3. General procedure for the synthesis of 4(a–j)

A solution of chloride **3a** (1.27 g, 5 mmol) in dry acetonitrile was added dropwise to a stirred solution of the corresponding amine in dry acetonitrile containing triethylamine (1.1 g, 11 mmol) over 30 min at room temperature. Stirring was continued for additional 3 h. Upon completion of the reaction, the solvent was evaporated under vacuum and the residue was treated with water and stirred for 1 h. The insoluble solid was filtered off and washed with water to provide the crude product, which was recrystallized from DMSO/H_2O.

4.2.3.1.1,2-(Bis-1,2-benzisoselenazol-3(2*H*)-one)ethane (4a). Compound **3a** and 1,2-diaminoethane (0.16 g, 2.6 mmol) were used as reactants to give 0.98 g of **4a** as yellow solid (92%). ^1H NMR (400 MHz, DMSO-d_6) δ 7.96 (d, J = 8.0, 2H, ArH), 7.80 (d, J = 7.7, 2H, ArH), 7.56 (t, J = 8.0, 2H, ArH), 7.39 (t, J = 7.7, 2H, ArH), 4.01 (s, 4H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.59, 139.69, 131,38, 127.65, 127.31, 125.82, 125.64, 43.12. HRMS Calcd for $C_{16}H_{12}N_2O_2Se_2$: 423.9229. Found 424.9302 (M^++H). HPLC t_R = 18.06 min (water/methanol (40:60), purity 99.99%).

4.2.3.2. 1,3-(Bis-1,2-benzisoselenazol-3(2*H*)-one)propane (4b). Compound **3a** and 1,3-diaminopropane (0.2 g, 2.7 mmol) were used as reactants to give 0.95 g of **4b** as yellow solid (87%). ^1H NMR (400 MHz, DMSO-d_6) δ 8.05 (d, J = 8.4, 2H, ArH), 7.80 (d, J = 7.6, 2H, ArH), 7.60 (t, J = 8.4, 2H, ArH), 7.41 (t, J = 7.6, 2H, ArH), 3.79 (t, J = 6.9, 4H, CH_2), 2.00 (quint, J = 6.9, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.89, 139.76, 131.92, 128.41, 127.77, 126.36, 126.24, 41.55, 31.55. HRMS Calcd for $C_{17}H_{14}N_2O_2Se_2$: 437.9386. Found 438.9461 (M^++H). HPLC t_R = 24.03 min (water/methanol (50:50), purity 98.87%).

4.2.3.3. 1,4-(Bis-1,2-benzisoselenazol-3(2*H*)-one)butane (4c). Compound **3a** and 1,4-diaminobutane (0.24 g, 2.7 mmol) were used as reactants to give 0.96 g of **4c** as yellow solid (85%). ^1H NMR (400 MHz, DMSO-d_6) δ 8.03 (d, J = 8.3, 2H, ArH), 7.79 (d, J = 7.3, 2H, ArH), 7.58 (t, J = 8.3, 2H, ArH), 7.40 (t, J = 7.3, 2H, ArH), 3.76 (s, 4H, CH_2), 1.66 (s,

4H,CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.27, 139.07, 131.33, 127.94, 127.26, 125.81, 125.68, 42.73, 27.06. HRMS Calcd for $C_{18}H_{16}N_2O_2Se_2$: 451.9542. Found 452.9618 (M^++H). HPLC t_R = 25.57 min (water/methanol (50:50), purity 96.22%).

4.2.3.4. 1,5-(Bis-1,2-benzisoselenazol-3(2*H*)-one)pentane (4d). Compound **3a** and 1,5-diaminopentane (0.29 g, 2.8 mmol) were used as reactants to give 0.98 g of **4d** as yellow solid (84%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.02 (d, J = 8.1, 2H, ArH), 7.74 (d, J = 7.3, 2H, ArH), 7.52 (t, J = 8.1, 2H, ArH), 7.41 (t, J = 7.3, 2H, ArH), 3.71 (t, J = 7.0, 4H, CH_2), 1.68 (quint, J = 7.0, 4H, CH_2), 1.35 (quint, J = 7.0, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.71, 139.54, 131.83, 128.52, 127.77, 126.29, 126.20, 43.57, 30.04, 23.77. HRMS Calcd for $C_{19}H_{18}N_2O_2Se_2$: 465.9699. Found 466.9785 (M^++H). HPLC t_R = 23.87 min (water/methanol (50:50), purity 99.45%).

4.2.3.5. 1,6-(Bis-1,2-benzisoselenazol-3(2*H*)-one)hexane (4e). Compound **3a** and 1,6-diaminohexane (0.30 g, 2.6 mmol) were used as reactants to give 1.03 g of **4e** as yellow solid (86%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.04 (d, J = 8.1, 2H, ArH), 7.84 (d, J = 7.6, 2H, ArH), 7.59 (t, J = 8.1, 2H, ArH), 7.44 (t, J = 7.6, 2H, ArH), 3.71 (t, J = 7.0, 4H, CH_2), 1.63 (quint, J = 7.0, 4H, CH_2), 1.36 (quint, J = 7.0, 4H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.69, 139.51, 131.83, 128.54, 127.77, 126.88, 126.21, 43.63, 30.31, 26.25. HRMS Calcd for $C_{20}H_{20}N_2O_2Se_2$: 479.9855. Found 480.9936 (M^++H). HPLC t_R = 37.26 min (water/methanol (50:50), purity 96.10%).

4.2.3.6. 1,7-(Bis-1,2-benzisoselenazol-3(2*H*)-one)heptane (4f). Compound **3a** and 1,7-diaminoheptane (0.38 g, 2.9 mmol) were used as reactants to give 0.99 g of **4f** as yellow solid (80%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.03 (d, J = 8.0, 2H, ArH), 7.79 (d, J = 7.5, 2H, ArH), 7.51 (t, J = 8.0, 2H, ArH), 7.40 (t, J = 7.5, 2H, ArH), 3.71 (t, J = 7.2, 4H, CH2), 1.63 (quint, J = 7.0, 4H, CH_2), 1.33 $\sim$ 1.28 (m, 6H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.15, 138.98, 131.31, 128.02, 127.25, 125.77, 125.69, 43.12, 29.81, 28.25, 26.01. HRMS Calcd for $C_{21}H_{22}N_2O_2Se_2$: 494.0012. Found 495.0085 (M^++H). HPLC t_R = 6.10 min (water/methanol (20:80), purity 96.37%).

4.2.3.7. 1,8-(Bis-1,2-benzisoselenazol-3(2*H*)-one)octane (4g). Compound **3a** and 1,8-diaminooctane (0.36 g, 2.5 mmol) were used as reactants to give 0.98 g of **4g** as yellow solid (77%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.03 (d, J = 8.0, 2H, ArH), 7.79 (d, J = 7.5, 2H, ArH), 7.51 (t, J = 8.0, 2H, ArH), 7.40 (t, J = 7.5, 2H, ArH), 3.70 (t, J = 7.3, 4H, CH_2), 1.61 (quint, J = 7.0, 4H, CH_2), 1.29 (s, 8H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.91, 137.98, 131.55, 128.88, 127.43, 125.12, 124.56, 44.42, 28.55, 27.15, 24.82. HRMS Calcd for $C_{22}H_{24}N_2O_2Se_2$: 508.0168. Found 509.0242 (M^++H). HPLC t_R = 7.08 min (water/methanol (20:80), purity 97.31%).

4.2.3.8. 1,6-(Bis-1,2-benzisoselenazol-3(2*H*)-one)cyclohexane (4h). Compound **3a** and 1,6-diamino- cyclohexane (0.33 g, 2.9 mmol) were used as reactants to give 0.85 g of **4h** as yellow solid (71%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.01 (d, J = 8.2, 2H, ArH), 7.65 (d, J = 7.3, 2H, ArH), 7.45 (t, J = 8.2, 2H, ArH), 7.33 (t, J = 7.3, 2H, ArH), 4.53 (s, 2H, CH), 2.10–2.00 (m, 8H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.20, 138.90, 133.06, 128.27, 127.37, 126.03, 125.84, 49.81, 29.15. HRMS Calcd for $C_{20}H_{18}N_2O_2Se_2$: 477.9699. Found 478.9764 (M^++H). HPLC t_R = 34.30 min (water/methanol (50:50), purity 95.58%).

4.2.3.9. α,4-(Bis-1,2-benzisoselenazol-3(2*H*)-one)toluene (4i). Compound **3a** and α,4-diaminotoluene (0.33 g, 2.7 mmol) were used as reactants to give 0.79 g of **4i** as yellow solid (65%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.09 (d, J = 8.0, 2H, ArH), 8.03 (d, J = 8.0, 2H, ArH), 7.89 (d, J = 7.7, 2H, ArH), 7.87 (d, J = 7.7, 2H, ArH), 7.70 $\sim$ 7.60 (m, 4H, ArH), 7.50 $\sim$ 7.40 (m, 4H, ArH), 4.94 (s, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.30, 164.95, 139.30, 138.90, 138.81, 135.82, 135.20, 132.20, 128.73, 127.88, 125.81, 124.63, 46.20. HRMS Calcd for $C_{21}H_{14}N_2O_2Se_2$: 485.9385. Found 486.9457 (M^++H). HPLC t_R = 5.27 min (water/methanol (20:80), purity 96.83%).

4.2.3.10. 1,4-(Bis-1,2-benzisoselenazol-3(2*H*)-one)benzene (4j). Compound **3a** and 1,4-diaminobenzene (0.28 g, 2.6 mmol) were used as reactants to give 0.89 g of **4j** as yellow solid (75%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.10 (d, J = 8.0, 2H, ArH), 7.91 (d, J = 7.7, 2H, ArH), 7.72 (s, 4H, ArH), 7.68 (t, J = 8.0, 2H, ArH), 7.49 (t, J = 7.7, 2H, ArH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.01, 138.90, 137.14, 132.27, 128.44, 127.92, 126.50, 125.27. HRMS Calcd for $C_{20}H_{12}N_2O_2Se_2$: 471.9229. Found 472.9301 (M^++H). HPLC t_R = 5.70 min (water/methanol (20:80), purity 95.14%).

4.2.4. General procedure for synthesis of 5(a–f), 6(a–e), 7(a, b), 8 (a, b), 9, 10(a, b), 11(a, b), 12, 13

A solution of chloride **3**(**b–k**) (5 mmol) in dry dichloromethane was added dropwise to a stirred solution of the corresponding amine in dry dichloromethane containing triethylamine (1.1 g, 11 mmol) over 30 min at room temperature. Stirring was continued for additional 4 h. Upon completion of the reaction, the solvent was evaporated under vacuum and the residue was treated with water and stirred for 1 h. The insoluble solid was filtered off and washed with water to give the crude product, which was recrystallized from DMSO/H_2O.

4.2.4.1. 1,2-(5,5′-Dimethyl(bis-1,2-benzisoselenazol-3(2*H*)-one)) ethane (5a). Compound **3b** (1.34 g, 5 mmol) and 1,2-diaminoethane (0.16 g, 2.6 mmol) were used as reactants to give 0.99 g of **5a** as yellow solid (88%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.82 (d, J = 8.2, 2H, ArH), 7.61 (d, J = 1.4, 2H, ArH), 7.39 (dd, J_1 = 8.2, J_2= 1.4, 2H, ArH), 3.97 (s, 4H, CH_2), 2.38 (s, 6H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.55, 136.42, 135.19, 132.70, 129.26, 127.38, 126.06, 125.68, 43.20, 20.57. MS (EI) m/z: 452.1 (M^+). HPLC t_R = 9.23 min (water/methanol (40:60), purity 99.58%).

4.2.4.2. 1,3-(5,5′-Dimethyl(bis-1,2-benzisoselenazol-3(2*H*)-one)) propane (5b). Compound **3b** (1.34 g, 5 mmol) and 1,3-diaminopropane (0.19 g, 2.6 mmol) were used as reactants to give 0.97 g of **5b** as yellow solid (83%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.90 (d, J = 8.1, 2H, ArH), 7.69 (d, J = 1.9, 2H, ArH), 7.43 (dd, J_1 = 8.1, J_2= 1.9, 2H, ArH), 3.75 (t, J = 7.0, 4H, CH_2), 2.39 (s, 6H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.33, 135.90, 135.21, 132.59, 127.90, 127.26, 125.56, 41.08, 31.00, 20.41. MS (EI) m/z: 466.0 (M^+). HPLC t_R = 15.90 min (water/methanol (40:60), purity 98.84%).

4.2.4.3. 1,4-(5,5′-Dimethyl(bis-1,2-benzisoselenazol-3(2*H*)-one)) butane (5c). Compound **3b** (1.34 g, 5 mmol) and 1,4-diaminobutane (0.23 g, 2.6 mmol) were used as reactants to give 0.95 g of **5c** as yellow solid (79%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.90 (d, J = 8.0, 2H, ArH), 7.72 (d, J = 1.8, 2H, ArH), 7.41 (dd, J_1 = 8.0, J_2= 1.8, 2H, ArH), 3.73 (s, 4H, CH_2), 2.38 (s, 6H, CH_3), 1.63 (s, 4H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.54, 134.65, 134.71, 131.88, 127.54, 127.76, 125.98, 42.79, 29.97, 20.34. HRMS Calcd for $C_{20}H_{20}N_2O_2Se_2$: 479.9855. Found 480.9923 (M^++H). HPLC t_R = 17.31 min (water/methanol (40:60), purity 97.96%).

4.2.4.4. 1,5-(5,5′-dimethyl(bis-1,2-benzisoselenazol-3(2*H*)-one)) pentane (5d). Compound **3b** (1.34 g, 5 mmol) and 1,5-diaminopentane (0.27 g, 2.6 mmol) were used as reactants to give 0.94 g of **5d**. as yellow solid (76%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.88 (d, J = 8.1, 2H, ArH), 7.60 (s, 2H, ArH), 7.41 (d, J = 8.1, 2H, ArH), 3.67 (t, J = 6.9, 4H, CH_2), 2.39 (s, 6H, CH_3), 1.65 (quint, J = 6.9, 4H, CH_2), 1.33 (quint, J = 6.9, 2H, CH_2). ^{13}C NMR (100 MHz,

DMSO-d_6) δ 166.66, 136.27, 135.69, 133.05, 128.54, 127.77, 126.01, 43.57, 30.03, 28.7, 20.92. MS (EI) *m/z*: 494.0 (M^+). HPLC t_R = 6.42 min (water/methanol (20:80), purity 96.94%).

4.2.4.5. 1,6-(5,5′-Dimethyl(bis-1,2-benzisoselenazol-3(2*H*)-one)) hexane (5e). Compound **3b** (1.34 g, 5 mmol) and 1,6-diaminohexane (0.30 g, 2.6 mmol) were used as reactants to give 1.04 g of **5e** as yellow solid (82%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.88 (d, J = 8.2, 2H, ArH), 7.62 (s, 2H, ArH), 7.39 (d, J = 8.2, 2H, ArH), 2.39 (s, 6H, CH_3), 3.70 (t, J = 6.9, 4H, CH_2), 1.60 (s, 4H, CH_2), 1.33 (s, 4H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.13, 135.63, 135.20, 132.53, 128.04, 127.26, 125.49, 43.11, 29.79, 25.71, 20.40. HRMS Calcd for $C_{22}H_{24}N_2O_2Se_2$: 508.0168. Found 509.0231 (M^++H). HPLC t_R = 7.44 min (water/methanol (20:80), purity 97.30%).

4.2.4.6. 1,4-(5,5′-Dimethyl(bis-1,2-benzisoselenazol-3(2*H*)-one)) cyclohexane (5f). Compound **3b** (1.34 g, 5 mmol) and 1,4-diaminocyclohexane (0.30 g, 2.6 mmol) were used as reactants to give 0.93 g of **5f** as yellow solid (74%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.90 (d, J = 8.4, 2H, ArH), 7.73 (s, 2H, ArH), 7.41 (d, J = 8.4, 2H, ArH), 4.49 (s, 2H, CH), 2.40 (s, 6H, CH_3), 2.02 $\sim$ 1.90 (m, 8H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.87, 135.21, 134.80, 132.78, 127.55, 126.87, 125.12, 49.82, 29.02, 20.42. MS (EI) *m/z*: 505.0 (M^+). HPLC t_R = 7.41 min (water/methanol (20:80), purity 98.14%).

4.2.4.7. 1,2-(5,5′-Difluoro(bis-1,2-benzisoselenazol-3(2*H*)-one)) ethane (6a). Compound **3c** (1.36 g, 5 mmol) and 1,2-diaminoethane (0.16 g, 2.6 mmol) were used as reactants to give 1.05 g of **6a** as yellow solid (91%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.98 (dd, J_1 = 8.8, J_2 = 4.9, 2H, ArH), 7.54 (dd, J_1 = 8.6, J_2 = 2.7, 2H, ArH), 7.48 (td J_1 = 8.8, J_2 = 2.7, 2H, ArH), 4.00 (s, 4H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.94, 160.48 (d, J_{C-F} = 249.1), 130.21 (d, J_{C-F} = 5.9), 128.34 (d, J_{C-F} = 6.9), 126.12 (d, J_{C-F} = 2.1), 120.45 (d, J_{C-F} = 22.1), 116.81 (d, J_{C-F} = 20.5), 43.21. HRMS Calcd for $C_{16}H_{10}N_2O_2F_2Se_2$: 459.9041. Found 460.9121 (M^++H). HPLC t_R = 4.72 min (water/methanol (20:80), purity 95.30%).

4.2.4.8. 1,3-(5,5′-Difluoro(bis-1,2-benzisoselenazol-3(2*H*)-one)) propane (6b). Compound **3c** (1.36 g, 5 mmol) and 1,3-diaminopropane (0.19 g, 2.6 mmol) were used as reactants to give 1.07 g of **6b** as yellow solid (90%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.05 (dd, J_1 = 8.7, J_2 = 5.1, 2H, ArH), 7.54 (dd, J_1 = 8.5, J_2 = 2.6, 2H, ArH), 7.48 (td J_1 = 8.7, J_2 = 2.6, 2H, ArH), 3.78 (t, J = 6.9, 4H, CH_2), 1.99 (quint, J = 6.9, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.53, 161.11 (d, J_{C-F} = 242.9), 134.35, 129.60 (d, J_{C-F} = 7.4), 127.94 (d, J_{C-F} = 9.5), 121.66 (d, J_{C-F} = 21.1), 113.00 (d, J_{C-F} = 23.3), 41.28, 30.96. MS (EI) *m/z*: 473.9 (M^+). HPLC t_R = 5.66 min (water/methanol (20:80), purity 97.44%).

4.2.4.9. 1,4-(5,5′-Difluoro(bis-1,2-benzisoselenazol-3(2*H*)-one))-butane (6c). Compound **3c** (1.36 g, 5 mmol) and 1,4-diaminobutane (0.23 g, 2.6 mmol) were used as reactants to give 1.11 of **6c** as yellow solid (91%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.04 (dd, J_1 = 8.8, J_2 = 4.8, 2H, ArH), 7.52 (dd, J_1 = 8.6, J_2 = 2.6, 2H, ArH), 7.49 (td J_1 = 8.8, J_2 = 2.6, 2H, ArH), 3.75 (s, 4H, CH_2), 1.65 (s, 4H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.29, 161.02 (d, J_{C-F} = 236.7), 133.96, 129.51 (d, J_{C-F} = 7.4), 127.73 (d, J_{C-F} = 7.4), 119.32 (d, J_{C-F} = 23.6), 112.83 (d, J_{C-F} = 22.8), 42.91, 26.89. MS (EI) *m/z*: 487.9 (M^+). HPLC t_R = 5.72 min (water/methanol (20:80), purity 97.74%).

4.2.5. 1,5-(5,5′-Difluoro(bis-1,2-benzisoselenazol-3(2*H*)-one))pentane (6d)

Compound **3c** (1.36 g, 5 mmol) and 1,5-diaminopentane (0.28 g, 2.7 mmol) were used as reactants to give 1.15 g of **6d** as yellow solid (92%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.04 (dd, J_1 = 8.8, J_2 = 5.0, 2H, ArH), 7.51 (dd, J_1 = 8.6, J_2 = 2.5, 2H, ArH), 7.47 (td J_1 = 8.8, J_2 = 2.5, 2H, ArH), 3.69 (t, J = 6.9, 4H, CH_2), 1.66 (quint, J = 6.9, 4H, CH_2), 1.33 (quint, J = 6.9, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.55, 162.12 (d, J_{C-F} = 241.2), 133.56, 128.41 (d, J_{C-F} = 6.9), 127.51 (d, J_{C-F} = 7.1), 119.96 (d, J_{C-F} = 21.5), 112.49 (d, J_{C-F} = 21.8), 43.24, 29.37, 23.13. MS (EI) *m/z*: 501.9 (M^+). HPLC t_R = 5.28 min (water/methanol (20:80), purity 97.89%).

4.2.5.1. 1,4-(5,5′-Difluoro(bis-1,2-benzisoselenazol-3(2*H*)-one)) cyclohexane (6e). Compound **3c** (1.36 g, 5 mmol) and 1,4-diaminocyclohexane (0.32 g, 2.8 mmol) were used as reactants to give 1.14 of **6e** as yellow solid (89%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.11 (dd, J_1 = 8.8, J_2 = 5.1, 2H, ArH), 7.58 (dd, J_1 = 8.7, J_2 = 2.7, 2H, ArH), 7.51 (td J_1 = 8.8, J_2 = 2.7, 2H, ArH), 4.51 (s, 2H, CH), 2.09 $\sim$ 2.00 (m, 8H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.97, 161.76 (d, J_{C-F} = 244.3), 132.59, 128.87 (d, J_{C-F} = 6.9), 126.88 (d, J_{C-F} = 7.7), 117.54 (d, J_{C-F} = 22.3), 112.61 (d, J_{C-F} = 22.1), 50.12, 28.89. MS (EI) *m/z*: 513.9 (M^+). HPLC t_R = 6.75 min (water/methanol (20:80), purity 97.44%).

4.2.5.2. 1,2-(5,5′-Dichloro(bis-1,2-benzisoselenazol-3(2*H*)-one)) ethane (7a). Compound **3d** (1.44 g, 5 mmol) and 1,2-diaminoethane (0.16 g, 2.6 mmol) were used as reactants to give 1.01 g of **7a** as yellow solid (82%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.98 (d, J = 8.6, 2H, ArH), 7.74 (d, J = 2.0, 2H, ArH), 7.62 (dd, J_1 = 8.6, J_2 = 2.0, 2H, ArH), 4.00 (s, 4H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.96, 141.51, 132.77, 131.87, 130.59, 129.27, 129.02, 41.86. MS (EI) *m/z*: 492.2 (M^+). HPLC t_R = 6.54 min (water/methanol (20:80), purity 99.17%).

4.2.5.3. 1,5-(5,5′-Dichloro(bis-1,2-benzisoselenazol-3(2*H*)-one)) pentane (7b). Compound **3d** (1.44 g, 5 mmol) and 1,5-diaminopentane (0.29 g, 2.8 mmol) were used as reactants to give 1.15 g of **7b** as yellow solid (86%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.06 (d, J = 8.5, 2H, ArH), 7.73 (d, J = 2.2, 2H, ArH), 7.65 (dd, J_1 = 8.5, J_2 = 2.2, 2H, ArH), 3.71 (t, J = 6.8, 4H, CH_2), 1.67 (quint, J = 6.8, 4H, CH_2), 1.33 (quint, J = 6.8, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.77, 142.54, 133.51, 132.00, 130.41, 129.85, 128.70, 43.20, 29.60, 23.43. MS (EI) *m/z*: 533.9 (M^+). HPLC t_R = 9.37 min (water/methanol (20:80), purity 97.15%).

4.2.5.4. 1,2-(5,5′-Dibromo(bis-1,2-benzisoselenazol-3(2*H*)-one)) ethane (8a). Compound **3e** (1.66 g, 5 mmol) and 1,2-diaminoethane (0.16 g, 2.7 mmol) were used as reactants to give 1.17 g of **8a** as yellow solid (81%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.91 (d, J = 8.6, 2H, ArH), 7.87 (s, 2H, ArH), 7.73 (d, J = 8.6, 2H, ArH), 4.00 (s, 4H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.98, 146.42, 139.15, 137.32, 131.77, 127.23, 121.45, 42.40. MS (EI) *m/z*: 581.7 (M^+). HPLC t_R = 7.39 min (water/methanol (20:80), purity 96.83%).

4.2.5.5. 1,5-(5,5′-Dibromo(bis-1,2-benzisoselenazol-3(2*H*)-one)) pentane (8b). Compound **3e** (1.66 g, 5 mmol) and 1,5-diaminopentane (0.28 g, 2.7 mmol) were used as reactants to give 1.31 g of **8b** as yellow solid (84%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.97 (d, J = 8.5, 2H, ArH), 7.84 (s, 2H, ArH), 7.75 (d, J = 8.5, 2H, ArH), 3.69 (t, J = 6.9, 4H, CH_2), 1.65 (quint, J = 6.9, 4H, CH_2), 1.31 (quint, J = 6.9, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.66, 145.48, 138.92, 137.11, 131.57, 127.40, 121.79, 42.24, 29.39, 22.21. MS (EI) *m/z*: 621.8 (M^+). HPLC t_R = 11.28 min (water/methanol (20:80), purity 97.50%).

4.2.5.6. 1,2-(5,5′-Dinitro(bis-1,2-benzisoselenazol-3(2*H*)-one)) ethane (9). Compound **3f** (1.5 g, 5 mmol) and 1,2-diaminoethane (0.16 g, 2.6 mmol) were used as reactants to give 0.95 g of **9** as orange solid (74%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.43 (d, J = 2.2, 2H, ArH), 8.36 (dd, J_1 = 8.8, J_2 = 2.2, 2H, ArH), 8.22 (d,

J = 8.8, 2H, ArH), 4.09 (s, 4H, CH2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.33, 147.92, 146.02, 128.49, 127.36, 125.11, 121.79, 43.10. MS (EI) m/z: 514.1 (M^+). HPLC t_R = 7.19 min (water/methanol (40:60), purity 95.25%).

4.2.5.7. 1,2-(4,4′-Difluoro(bis-1,2-benzisoselenazol-3(2*H*)-one)) ethane (10a). Compound **3g** (1.36 g, 5 mmol) and 1,2-diaminoethane (0.16 g, 2.6 mmol) were used as reactants to give 0.92 g of **10a** as yellow solid (80%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.82 (dd, J_1 = 8.5, J_2 = 5.5, 2H, ArH), 7.74 (dd, J_1 = 9.2, J_2 = 2.3, 2H, ArH), 7.24 (td, J_1 = 8.5, J_2 = 2.3, 2H, ArH), 3.99 (s, 4H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.65, 161.54 (d, J_{C-F} = 224.5), 141.62 (d, J_{C-F} = 8.8), 129.33 (d, J_{C-F} = 9.9), 124.37 (d, J_{C-F} = 1.4), 113.88 (d, J_{C-F} = 23.4), 112.20 (d, J_{C-F} = 26.3), 43.06. MS (EI) m/z: 460.0 (M^+). HPLC t_R = 5.36 min (water/methanol (20:80), purity 98.74%).

4.2.5.8. 1,5-(4,4′-Difluoro(bis-1,2-benzisoselenazol-3(2*H*)-one)) pentane (10b). Compound **3g** (1.36 g, 5 mmol) and 1,5-diaminopentane (0.27 g, 2.6 mmol) were used as reactants to give 0.97 g of **10b** as yellow solid (77%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.83 (dd, J_1 = 8.5, J_2 = 5.6, 2H, ArH), 7.69 (dd, J_1 = 9.2, J_2 = 2.2, 2H, ArH), 7.21 (td, J_1 = 8.5, J_2 = 2.2, 2H, ArH), 3.68 (t, J = 6.9, 4H, CH_2), 1.65 (quint, J = 6.9, 4H, CH_2), 1.32 (quint, J = 6.9, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.32, 163.87 (d, J_{C-F} = 248.8), 141.11 (d, J_{C-F} = 11.1), 129.33 (d, J_{C-F} = 10.2), 124.83, 114.01 (d, J_{C-F} = 23.37), 112.32 (d, J_{C-F} = 26.95), 43.07, 29.45, 23.20. MS (EI) m/z: 502.0 (M^+). HPLC t_R = 5.38 min (water/methanol (20:80), purity 97.00%).

4.2.5.9. 1,2-(4,4′-Dichloro(bis-1,2-benzisoselenazol-3(2*H*)-one)) ethane (11a). Compound **3h** (1.44 g, 5 mmol) and 1,2-diaminoethane (0.16 g, 2.6 mmol) were used as reactants to give 0.92 g of **11a** as yellow solid (75%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.06 (d, J = 1.8, 2H, ArH), 7.75 (d, J = 8.3, 2H, ArH), 7.44 (dd, J_1 = 8.3, J_2 = 1.8, 2H, ArH), 3.69 (s, 4H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.62, 150.30, 135.11, 133.90, 130.88, 127.74, 121.59, 44.01. MS (EI) m/z: 492.0 (M^+). HPLC t_R = 7.83 min (water/methanol (20:80), purity 96.52%).

4.2.5.10. 1,5-(4,4′-Dichloro(bis-1,2-benzisoselenazol-3(2*H*)-one))pentane (11b). Compound **3h** (1.44 g, 5 mmol) and 1,5-diaminopentane (0.27 g, 2.6 mmol) were used as reactants to give 1.03 g of **11b** as yellow solid (77%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.07 (s, 2H ArH), 7.75 (d, J = 7.8, 2H, ArH), 7.44 (d, J = 7.8, 2H, ArH), 3.68 (t, J = 6.8, 4H, CH_2), 1.65 (quint, J = 6.8, 4H, CH_2), 1.31 (quint, J = 6.8, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.33, 150.87, 135.55, 133.51, 131.23, 126.91, 120.32, 43.56, 33.20, 26.51. MS (EI) m/z: 533.9 (M^+). HPLC t_R = 8.05 min (water/methanol (20:80), purity 97.11%).

4.2.5.11. 1,2-(5,5′-Dimethoxy(bis-1,2-benzisoselenazol-3(2*H*)-one))ethane (12). Compound **3j** (1.42 g, 5 mmol) and 1,2-diaminoethane (0.16 g, 2.6 mmol) were used as reactants to give 0.88 g of **12** as yellow solid (73%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.82 (d, J = 8.8, 2H, ArH), 7.29 (d, J = 2.7, 2H, ArH), 7.20 (dd, J_1 = 8.8, J_2 = 2.7, 2H, ArH), 3.98 (s, 4H, CH_2), 3.80 (s, 6H, OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.36, 158.14, 130.49, 128.66, 127.00, 120.49, 109.77, 55.50, 43.51. HRMS Calcd for $C_{18}H_{16}N_2O_4Se_2$: 483.9441. Found 484.9558 (M^++H). HPLC t_R = 4.81 min (water/methanol (20:80), purity 96.98%).

4.2.5.12. 1,2-(5,5′-Diacetoxy(bis-1,2-benzisoselenazol-3(2*H*)-one))ethane (13). Compound **3k** (1.56 g, 5 mmol) and 1,2-diaminoethane (0.16 g, 2.7 mmol) were used as reactants to give 1.19 g of **13** as white solid (88%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.97 (d, J = 8.6, 2H, ArH), 7.55 (d, J = 2.5, 2H, ArH), 7.35 (dd, J_1 = 8.6, J_2 = 2.5, 2H, ArH), 4.01 (s, 4H, CH_2), 2.28 (s, 6H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.29, 165.89, 148.76, 136.40, 128.67, 126.88, 125.58, 120.08, 43.18, 20.79. HRMS Calcd for $C_{20}H_{16}N_2O_6Se_2$: 539.9339. Found 562.9232 (M^++Na). HPLC t_R = 6.90 min (water/methanol (40:60), purity 96.29%).

4.2.5.13. 1,2-(5,5′-Dihydroxy(bis-1,2-benzisoselenazol-3(2*H*)-one))ethane (14). Compound **13** (2 g, 4.5 mmol) was dissolved in 160 ml DMSO containing 32 ml HCl. The mixture was stirred for 2 h at 60 °C then poured into 900 ml icy water and filtrated under vacuum to give crude product which was recrystallized by DMSO and water to give 1.35 g of **14**. White solid (66%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.69 (s, 2H, OH), 7.72 (d, J = 8.6, 2H, ArH), 7.18 (d, J = 2.6, 2H, ArH), 7.04 (dd, J_1 = 8.6, J_2 = 2.6, 2H, ArH), 3.93 (s, 4H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.40, 156.05, 128.67, 128.16, 126.60, 120.56, 112.46, 43.24. HRMS Calcd for $C_{16}H_{12}N_2O_4Se_2$: 455.9128. Found 456.9196 (M^++H). HPLC t_R = 4.78 min (water/methanol (40:60), purity 98.39%).

4.3. Biology

TrxR of rat liver was prepared and purified according to the method of Luthman and Holmgren.[27] Trx of *Escherichia coli,* GR and DTNB were purchased from Sigma–Aldrich. BIAM was purchased from Invitrogen.

4.3.1. Inhibition of TrxR in vitro

DTNB assay was used to determine the TrxR inhibitory activity of the compounds tested. All assays were conducted at room temperature in 96-well microplates with a total volume of 80 μl. Twenty microliter of TrxR (10 μg) was added to 50 μl assay solution containing 40 μl of 100 mM potassium phosphate, 2 mM EDTA, pH 7.4 and 10 μl of 100 μM NADPH. Then 10 μl of inhibitors at various concentrations were added to each well followed by a five minute pre-incubation period. The same amounts of DMSO were added to the control experiments. The reaction was initiated by the addition of 100 μl DTNB and TrxR activities were measured by the increase of absorbance at 412 nm over the initial 120 s.

4.3.2. Cell culture and growth inhibition assay

Human cancer cell lines U87 MG (glioma), MIA PaCa-2 (pancreatic carcinoma), A549 (lung cancer) were cultured in DMEM medium and LoVo (colon cancer) was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100U/ml streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. Exponentially growing cells were then detached from the cell culture to form a single cell suspension. Cells were then plated into 96-well plates with 4000–5000 cells per well. Compounds dissolved in DMSO at different concentrations (0–50 μM) were added and incubated for 48 h. The effects of selected compounds on the growth of human cancer cell lines were determined by MTT assay as described.[23]

4.3.3. BIAM assay

The BIAM assay was conducted according to the literature[8] using a minor modification. Generally, NADPH-reduced TrxR (1.1 μM) and **12** with different concentrations were incubated separately at room temperature for 2 h. The control was incubated with the same amount of DMSO. Next, 2 μl of the reaction mixtures were taken out and added to new tubes containing 100 μM BIAM (pH 6.5 and 8.5) following incubation at 37 °C for 30 min. 40 μl of BIAM-modified enzymes were mixed with 40 μl loading buffer, then 40 μl of the samples were subjected to SDS-PAGE on a 8.0% gel, and the separated proteins were transferred to nitro cellulose membrane. Proteins labeled with BIAM were detected with horseradish peroxidase-conjugated streptavidin and enhanced chemiluminescence detection.

4.3.4. Cellular TrxR inhibition assay

U87 MG cells were incubated with different concentrations of selected compounds for 12 h. The same amount of DMSO (1%, v/v) was added to the control group. Then cells were washed with phosphate-buffered saline and lysed with cell lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 10 mM EDTA, 0.1% SDS and 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 13000*g* for 30 min at 4 °C. Protein concentration was determined by BCA method.[38] To determine the cellular TrxR activity, insulin-reducing assay was employed as shown in our previous study.[21] with minor modification. Briefly, 40 μg of protein in the cell extracts were incubated with reaction buffer (0.2 M HEPES, pH 7.6, 5 mM EDTA, 1 mM NADPH, 1 μM *E. coli* Trx and 2.5 mg/ml bovine insulin) for half an hour at 37 °C. Reactions were then quenched by the sequential addition of 120 μl of 6 M guanidine-HCl, 50 mM Tris (pH 8.0), and 0.6 mg/ml DTNB. The amount of free thiols generated from insulin reduction was measured by recording the absorbance at 412 nm. For each test sample, the corresponding control was measured by the same assay but without the addition of *E. coli* Trx. TrxR activity was calculated after subtracting the control value.

4.4. Docking Study

Structure of human thioredoxin reductase (PDB code: 2J3N) obtained from the Brookhaven Protein Database (www.rcsb.org/pdb) was applied as receptor model for the docking procedure in the Discovery Studio 2.5 software (Accelrys). Co-crystallized water was removed and hydrogen atoms were added. The receptor protein was then typed by applying the CHARMm forcefield before following a three-step minimization protocol (steepest descent, conjugate gradient and adopted basis Newton–Rapheson), while the ligand to be docked was first drawn by ChemDraw software and then was prepared for the docking simulation by employing the Prepare Ligands application in Discovery Studio 2.5. The prepared ligand was docked into the active site of TrxR by means of GOLD 3.0.1.[39,40] The site sphere was defined as 15 Å with selection of Cys 497, Sec 498, Cys 59 and Cys 64 (redox-active centers in C-terminal and N-terminal respectively). In an attempt to stimulate the formation of a covalent bond between two selenium atoms of **12** and Cys497 and Sec498, respectively, two distance constraints of the two pairs of atoms were made: one was applied as 2.11 Å (minimum) to 2.57 Å (maximum) around an average Se–Se distance[35] of 2.34 Å between one selenium atom in the inhibitor and the selenium atom of Sec498; another was applied as 2.70 Å (minimum) to 3.30 Å (maximum) between the other selenium atom in the inhibitor and the sulfur atom of Cys497 to allow their interactions.[3] Parameters were kept as default values and 10 genetic algorithm runs were applied in terms of GoldScore fitness function.

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Supplementary data

Supplementary data (synthetic procedures and analytical data for **15** and **16**; inhibitory effects of **12**, **4g**, **13** on GR.) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.04.033.

References and notes

1. Arnér, E. S. J.; Holmgren, A. *Eur. J. Biochem.* **2000**, *267*, 6102.
2. Nakamura, H. *Antioxid. Redox Signal.* **2005**, *7*, 823.
3. Sandalova, T.; Zhong, L.; Lindqvist, Y.; Holmgren, A.; Schneider, G. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 9533.
4. Zhong, L.; Arnér, E. S. J.; Holmgren, A. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 5854.
5. Lillig, C. H.; Holmgren, A. *Antioxid. Redox. Sign.* **2007**, *9*, 25.
6. Grogan, T. M.; Fenoglio-Prieser, C.; Zeheb, R.; Bellamy, W.; Frutiger, Y.; Vela, E.; Stemmerman, G.; Macdonald, J.; Richter, L.; Gallegos, A.; Powis, G. *Hum. Pathol.* **2000**, *31*, 475.
7. Smart, D. K.; Ortiz, K. L.; Mattson, D.; Bradbury, C. M.; Bisht, K. S.; Sieck, L. K.; Brechbiel, M. W.; Gius, D. *Cancer Res.* **2004**, *64*, 6716.
8. Fang, J.; Lu, J.; Holmgren, A. *J. Biol. Chem.* **2005**, *280*, 25284.
9. Arnér, E. S. J. *Biochim. Biophys. Acta.* **2009**, *1790*, 495.
10. Krishnamurthy, D.; Karver, M. R.; Fiorillo, E.; Stanford, S. M.; Bottini, N.; Barrios, A. M. *J. Med. Chem.* **2008**, *51*, 4790.
11. Rubbiani, R.; Kitanovic, I.; Alborzinia, H.; Can, S.; Kitanovic, A.; Onambele, L. A.; Stefanopoulou, M.; Geldmacher, Y.; Sheldrick, W. S.; Wolber, G.; Prokop, A.; Ott, I. *J. Med. Chem.* **2010**, *53*, 8608.
12. Lo, Y. C.; Ko, T. P.; Su, W. C.; Su, T. L.; Wang, A. H. J. *J. Inorg. Biochem.* **2009**, *103*, 1082.
13. Casini, A.; Gabbiani, C.; Sorrentino, F.; Rigobello, M. P.; Bindoli, A.; Geldbach, T. J.; Marrone, A.; Re, N.; Hartinger, C. G.; Dyson, P. J.; Messori, L. *J. Med. Chem.* **2008**, *51*, 6773.
14. Qiu, X.; Liu, Z.; Shao, W. Y.; Liu, X.; Jing, D. P.; Yu, Y. J.; An, L. K.; Huang, S. L.; Bu, X. Z.; Huang, Z. S.; Gu, L. Q. *Bioorg. Med. Chem.* **2008**, *16*, 8035.
15. Zhou, C.; Boggess, J. F.; Bae-Jump, V.; Gehrig, P. A. *Gynecol. Oncol.* **2007**, *105*, 218.
16. Cenas, N.; Nivinskas, H.; Anusevicius, Z.; Sarlauskas, J.; Lederer, F.; Arnér, E. S. J. *J. Biol. Chem.* **2004**, *279*, 2583.
17. Mehta, M. P.; Shapiro, W. R.; Phan, S. C.; Gervais, R.; Carrie, C.; Chabot, P.; Patchell, R. A.; Glantz, M. J.; Recht, L.; Langer, C.; Sur, R. K.; Roa, W. H.; Mahe, M. A.; Fortin, A.; Nieder, C.; Meyers, C. A.; Smith, J. A.; Miller, R. A.; Renschler, M. F. *Int. J. Radiat. Oncol.* **2009**, *73*, 1069.
18. Ramanathan, R.; Abbruzzese, J.; Dragovich, T.; Kirkpatrick, L.; Guillen, J.; Baker, A.; Pestano, L.; Green, S.; Von Hoff, D. *Cancer Chemother. Pharmacol.* **2011**, *67*, 503.
19. Hashemy, S. I.; Ungerstedt, J. S.; Avval, F. Z.; Holmgren, A. *J. Biol. Chem.* **2006**, *281*, 10691.
20. Baker, A. F.; Dragovich, T.; Tate, W. R.; Ramanathan, R. K.; Roe, D.; Hsu, C.-H.; Kirkpatrick, D. L.; Powis, G. *J. Lab. Clin. Med.* **2006**, *147*, 83.
21. Zhao, F.; Yan, J.; Deng, S.; Lan, L.; He, F.; Kuang, B.; Zeng, H. *Cancer Lett.* **2006**, *236*, 46.
22. Xing, F.; Li, S.; Ge, X.; Wang, C.; Zeng, H.; Li, D.; Dong, L. *Oral Oncol.* **2008**, *44*, 963.
23. Yin, H.; Li, J.; Xiong, K.; Wang, L.; Wang, T.; Tan, Q.; Fu, J.; Ren, X.; Zeng, H. *Differentiation* **2011**, *81*, 49.
24. Wang, L.; Fu, J.; Wang, J.; Jin, C.; Ren, X.; Tan, Q.; Li, J.; Yin, H.; Xiong, K.; Wang, T.; Liu, X.; Zeng, H. *Anti-Cancer Drugs* **2011**, *22*, 732.
25. Ninomiya, M.; Garud, D. R.; Koketsu, M. *Coordin. Chem., Rev.* **2011**, *255*, 2968.
26. Wang, L.; Yang, Z.; Fu, J.; Yin, H.; Xiong, K.; Tan, Q.; Jin, H.; Li, J.; Wang, T.; Tang, W.; Yin, J.; Cai, G.; Liu, M.; Kehr, S.; Becker, K.; Zeng, H. *Free Radical Biol. Med.* **2012**, *52*, 898.
27. Luthman, M.; Holmgren, A. *Biochemistry* **1982**, *21*, 6628.
28. Sakurai, T.; Kanayama, M.; Shibata, T.; Itoh, K.; Kobayashi, A.; Yamamoto, M.; Uchida, K. *Chem. Res. Toxicol.* **2006**, *19*, 1196.
29. Schöneich, C.; Narayanaswami, V.; Asmus, K. D.; Sies, H. *Arch. Biochem. Biophys.* **1990**, *282*, 18.
30. Kim, J. R.; Yoon, H. W.; Kwon, K. S.; Lee, S. R.; Rhee, S. G. *Anal. Biochem.* **2000**, *283*, 214.
31. Stadtman, T. C. *Ann. Rev. Biochem.* **1996**, *65*, 83.
32. Besse, D.; Siedler, F.; Diercks, T.; Kessler, H.; Moroder, L. *Angew. Chem., Int. Ed.* **1997**, *36*, 883.
33. Fang, J.; Holmgren, A. *J. Am. Chem. Soc.* **1879**, *2006*, 128.
34. Fritz-Wolf, K.; Urig, S.; Becker, K. *J. Mol. Biol.* **2007**, *370*, 116.
35. Allen, F. H.; Kennard, O.; Watson, D. G. *J. Chem. Soc., Perkin. Trans. II* **1987**, S1.
36. Gromer, S.; Wessjohann, L. A.; Eubel, J.; Brandt, W. *ChemBioChem* **2006**, *7*, 1649.
37. Cheng, Q.; Sandalova, T.; Lindqvist, Y.; Arnér, E. S. J. *J. Biol. Chem.* **2009**, *284*, 3998.
38. Morton, R. E.; Evans, T. A. *Anal. Biochem.* **1992**, *204*, 332.
39. Jones, G.; Willett, P.; Glen, R. C.; Leach, A. R.; Taylor, R. *J. Mol. Biol.* **1997**, *267*, 727.
40. Verdonk, M. L.; Cole, J. C.; Hartshorn, M. J.; Murray, C. W.; Taylor, R. D. *Proteins Struct., Funct., Bioinf.* **2003**, *52*, 609.