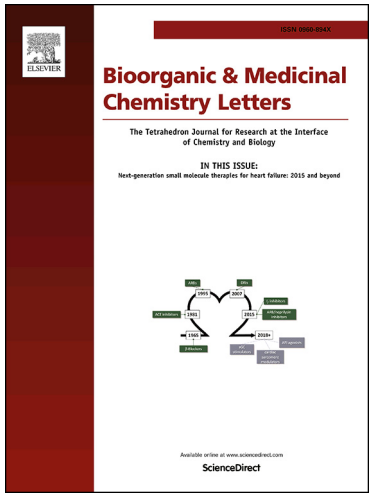

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Synthesis and comparison of substituted 1,2,3-dithiazole and 1,2,3-thiaselenazole as inhibitors of the feline immunodeficiency virus (FIV) nucleocapsid protein as a model for HIV infection

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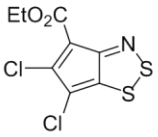
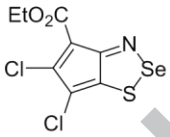
Compound 10c
CrFK - CC_{50} = >100 µM
FL-4 - CC_{50} = 3.5 µM
FL-4 - EC_{50} = 0.31 µM
TI - 11.4





Compound 11c
CrFK - CC_{50} = >100 µM
FL-4 - CC_{50} = 5.1 µM
FL-4 - EC_{50} = 0.082 µM
TI - 61.9

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Synthesis and comparison of substituted 1,2,3-dithiazole and 1,2,3-thiaselenazole as inhibitors of the feline immunodeficiency virus (FIV) nucleocapsid protein as a model for HIV infection

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ABSTRACT

We report the first biological evaluation the 1,2,3-thiaselenazole class of compound and utilising a concise synthetic approach of sulfur extrusion, selenium insertion of the 1,2,3-dithiazoles. We created a small diverse library of compounds to contrast the two ring systems. This approach has highlighted new structure activity relationship insights and lead to the development of sub-micro molar anti-viral compounds with reduced toxicity. The 1,2,3-thiaselenazole represents a new class of potential compounds for the treatment of FIV and HIV.

Continued innovation in the development of novel therapeutics and targets is essential in the fight against resistant strains of both viruses and bacteria. The human immunodeficiency virus (HIV) is an archetypal example, with an array of drugs and targets working at multiple points during the viral life cycle. However, the continual development of resistance has eroded the efficacy of existing drugs.[1]

Among the non-primates, lentiviruses only infect cats as a natural host. Feline immunodeficiency virus (FIV), causes an immunodeficiency in this animal resembling acquired immune deficiency syndrome (AIDS) disease in humans. This includes $CD4^+$ T lymphocyte depletion, central nervous system involvement and analogous AIDS-like disease progression, all hallmarks of HIV/AIDS.[2-4]

One under-explored anti-viral target with no clinically approved compounds, is the short basic nucleic acid binding nucleocapsid protein (NCp) present in both FIV and HIV. This lack of clinical compounds is surprising despite the NCp involvement at multiple points of the viral replication cycle including reverse transcription,[5-6] integration and the promotion of dimerization within freshly created virons.[7-9] The NCp contains a double zinc finger unit $C\text{-}X_2\text{-}C\text{-}X_4\text{-}H\text{-}X_4\text{-}C$ (CCHC) and these motifs are known to chelate Zn^{2+} ions with subpicomolar affinities through their cysteine and histidine residues.[10] The NCp presence in nearly all including HIV-1/2, FIV, Simian Immunodeficiency Virus (SIV), Equine Infectious Anemia Virus (EIAV) makes it a very attractive cross-species target.[11-15] Deletion or modification of either zinc finger leads to virus inactivation and inhibition yielding non-infectious virons.[16-17]

Two different approaches have been employed in the development of NCp inhibitors, one focused on competition with the binding of the substrate on the nucleic acid chain and the second focused on irreversible ejection of the structural Zn^{2+} ion.[18-19] The zinc ejection mechanism is a permanent modification and would provide a useful asset to be used in tandem with other HARRT medications.

Several advanced small molecules have been reported (**Fig. 1**) and one of the first compounds identified was 3-nitrosobenzamide – NOBA (**1**) which showed complete zinc extrusion together with virus inactivation.[20-21] This was followed with by the identification of (*E*)-diazene-1,2-dicarboxamide - ADA (**2**), that was progressed to clinical trails in 1997, 2001, and 2007, but development was discontinued.[22-24] The dithiane (**3**) chemotype was discovered as part of an NIH screening program and demonstrated an ability to extrude zinc *in vitro*.[25-26] Other chemotypes known as a zinc ejector of NCp7 include the pyridinioalkanoyl thioester (PATE) and the dithio*bis*(benzamides) DIBA class exemplified by **4** among others.[18,27-28] DIBA-4 (**4**) was progressed to pre-clinical investigations, however it was the

benzisothiazolone **5** that was investigated in phase 1 clinical trails. Dose dependent toxicity and poor serum stability culminated in the withdrawal of **5** from further development.[29]

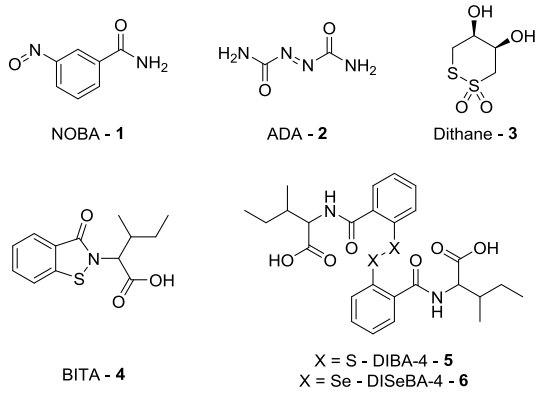


Figure 1. Previously reported NCp7 small molecule zinc ejectors.

A principle interest of our work has been the reactivity of internal disulfide bridge compounds and their medicinal chemistry application.[30] The high density poly-sulfide heterocycles lend themselves to this type of zinc ejection redox type application. We recently reported studies relating potent FIV inhibition profiles to *bis*[1,2]dithiolo[1,4]thiazines and *bis*[1,2]dithiolopyrrole derivatives, tetrathiocine and 1,2,3-dithiazoles derivatives.[31-33] We were curious if a more liable disulfide bridge on a similar 1,2,3-dithiazoles scaffold would have a greater propensity for zinc ejection without an increase in toxicity such as BITA (**4**). We were encouraged by a report of a successful selenide isosteric replacement to DIBA-4 (**5**) to DISeBA-4 (**6**) substituted HIV inhibitors with only very limited associated toxicity.[34]

We utilized an *in silico* homology model consisting of the well-defined HIV-1 and EIAV nucleocapsid proteins and selected a series of compounds to pursue based around the rare 1,2,3-thiaselenazoles heterocycle. Substitutions and electronic effects were then probed to explore their effects on the propensity of the disulfide to react with the cysteine thiolates of the NCp model. The compounds were the analyzed by using both molecular docking and DFT calculations (Fig. 2).[35]

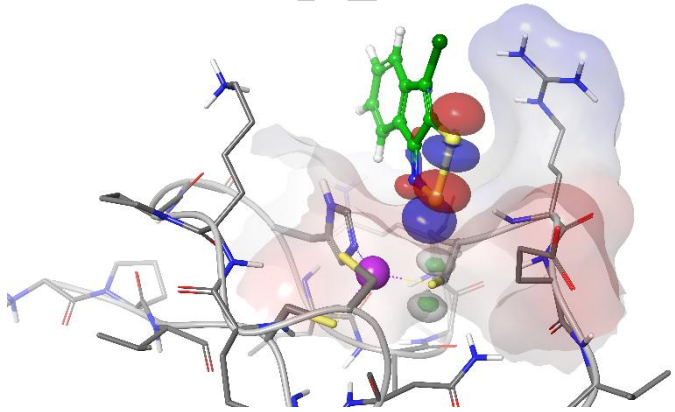
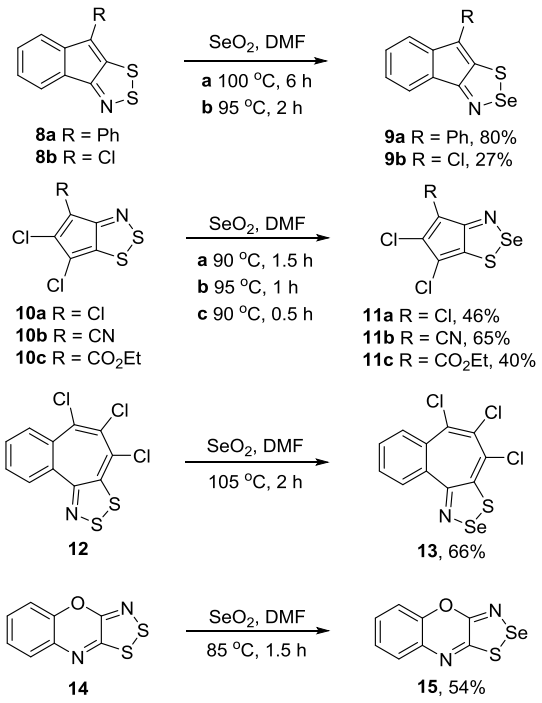


Figure 2. Docking example of Compound **8b** manually docked above QM based Zinc-model optimized with Jaguar (B3-LYP//MSV).[34] atomic fukui indices shown in white-green (isovalues 0.008 and 0.02).

The atomic fukui indices demonstrate the ability to set up a nucleophilic attack and highlight the best cysteine for this reaction. Compound **8b** LUMO orbitals are also shown indicating the most favourable direction of nucleophilic attack as well as bond breaking within the inhibitor. A set of compounds was synthesized using this model and tested (Tab. 1). We now report on the development of a series of selected 1,2,3-thiaselenazoles. This heterocycle is a very rare ring system[36] with only two reports in the literature.[37-38]

This chemotype poses a significant synthetic challenge. To access the desired 1,2,3-thiaselenazoles we choose to utilize a sulfur extrusion, selenium insertion strategy from the corresponding 1,2,3-dithiazoles (**Sch. 1**).[38] These 1,2,3-dithiazoles were created by a functionalized oxime intermediates.[39-40] The 1,2,3-dithiazoles were reacted with 10 equivalents of selenium dioxide in dimethylformamide (DMF) between 80-110 °C. There is no conversation at lower temperatures (<80 °C). However, selenium dioxide decomposes with prolonged heating in the reaction mixtures with DMF at the temperature about 100 °C, so a careful optimization is required for each substrate. Despite reaction condition screening including both solvent and temperature, excess selenium dioxide was required to compensate for decomposition under these relatively harsh conditions of DMF at about 100 °C.[38]

The conversion of 8-phenylindeno[1,2-*d*]-1,2,3-dithiazole (**8a**), to the corresponding 1,2,3-thiaselenazole (**9a**) proceeded in high yield. The chlorine derivative (**8b**) gave a much lower yield of 1,2,3-thiaselenazole (**9b**) likely due to homo-coupling through the chlorine atom. Treatment of 1,2,3-dithiazoles (**10a-c**) under similar conditions furnished the corresponding 1,2,3-thiaselenazole (**11a-c**) in moderate to good yields, with highly tuned reaction conditions. The conversion **12** to **13** required a slightly higher temperature (105 °C), while **14** to **15** was achieved at a much more modest 85 °C.



Scheme 1. Synthetic route to selenium derivatives from 1,2,3-dithiazoles.

The compounds were then screened for cytotoxicity against feline kidney cells and tested for anti-viral efficacy in a chronically infected feline lymphoid cell line with a corresponding cytotoxicity assay. We first screened the compounds in a short toxicity assay over 24 h, exposing Crandell Rees Feline Kidney cells (CrFK) to six concentrations (1 nM - 100 μM) of the compounds with an MTT assay used to quantify the level of cell viability.[41] The second stage gave an enhanced longer-term cytotoxicity screen and an anti-FIV profile that was realised over seven days at six concentrations ranging between 100 μM and

1 nM, using an IL-2 independent feline lymphoblastoid cell line (FL-4).

FL-4 cells infected with FIV were exposed to the compounds over seven days and sampled each day and at each of the six concentrations. To determine the extent of viral replication, viral RNA was isolated from cell culture supernatants in a MagNA Pure LC System using the Total Nucleic Acid Isolation Kit (Roche Applied Science, Switzerland). The supernatants were subsequently used to determine the viral load by a quantitative real-time reverse-transcription polymerase chain reaction (RT-qPCR) for FIV RNA.[42] The screening results were also checked for their viability using the MTT assay to rule out any toxicity effects validating the RT-qPCR result.

The 8-phenylindeno[1,2-*d*]-1,2,3-dithiazole (**8a**) showed the weakest of all anti-viral activity of all the tested compounds (**Tab. 1**). However, the 1,2,3-thiaselenazole analog (**9a**) showed a 100-fold increase in anti-viral efficacy with a small increase in cytotoxicity. The melting point of **9a** increased by approximately 90 °C over **8a** indicating this is likely not driven by solubility.[38-39] The corresponding chlorine derivative (**8b**) gave an 18-fold increase over **8a**; however the selenium analog (**9b**) showed no improvement in anti-viral efficacy but did show a 10-fold increase in toxicity in FL-4 cells. 1,2,3-dithiazole **10a** and 1,2,3-thiaselenazole **11a** also showed equi-potent anti-viral profiles, with no difference in toxicity. The switch to the cyano (**10b**) showed a 6-fold improvement in toxicity but no difference in anti-viral efficacy at just over EC_{50} = 1 µM. The selenium analog (**11b**) was just a touch less potent at EC_{50} = 3.8 µM and with a reduced melting point of over 40 °C this is likely unrelated to solubility. However, after a switch to a more water soluble ethyl ester (**10c** and **11c**) we observed a jump in potency in both the 1,2,3-dithiazole (**10c**) and 1,2,3-thiaselenazole (**11c**) with a 5-fold increase over **10a** and a 15-fold increase in anti-viral potency over **11a** when compared to the chlorine analogs.

Table 1. Results of toxicity and viral loading studies

Cmpd	CrFK	FL-4	EC_{50} (µM)[a]	TI[b]
	CC_{50} (µM)			
8a	>100	>100	25.7	>4
9a	56	50	0.26	193.6
8b	>100	49	1.4	36.3
9b	98	5.7	1.2	4.6
10a	68	6.1	1.6	3.8
11a	62	6.5	1.2	5.4
10b	>100	39	1.5	26.8
11b	>100	18	3.8	4.7
10c	>100	3.5	0.31	11.4
11c	>100	5.1	0.082	61.9
12	>100	9.3	3.0	3.1
13	>100	0.83	0.25	3.3
14	>100	57	4.0	14.0
15	>100	5.8	0.24	24.0
AZT	>100	>100	2.7	>37

[a]Geometric mean, each concentration tested in triplicate after 7 days as a difference of the untreated FL-4 cells. [b]Therapeutic index is CC_{50}/EC_{50}, which is the ratio of toxicity to activity.

The 7-membered ring of 4,5,6-trichlorobenzo[6,7]cyclohepta[1,2-*d*][1,2,3]dithiazole (**12**) produced a moderately active anti-viral compound at EC_{50} = 3.0 µM, however the switch to selenium (**13**) boosted activity by 12-fold. There was a dose dependant increase in toxicity in the case of **13** but this was mitigated with **14** and **15**.

Benzo[*b*][1,2,3]dithiazolo[5,4-*e*][1,4]oxazine (**14**) showed modest activity and the selenium heterocycle (**15**) showed a 17-fold increase in potency not matched by the toxicity seen in **13**. The activity of AZT is consistent with previous reports in FIV/HIV.[43]

Our proposed mechanism of action for this class of compound is similar to previously reported NMR and MS studies on HIV NCp7 that have shown observable formation of protein-zinc-thiol(ate) complexes and covalent modifcations.[44] We reasoned that 1,2,3-dithiazole/1,2,3-thiaselenazole mediated zinc ion ejection also occurs *via* an analogous mechanism to known zinc disrupting compounds, where a zinc-binding cysteinyl thiol(ate) reacts with the internal disulfide to generate a transient protein-compound disulfide (Fig. 3).[44-45] This likely then rearranges to form an intramolecular protein disulfide with consequent reduction in zinc ion affinity. The ejected zinc ion (or zinc 1,2,3-dithiazole/1,2,3-thiaselenazole complex) could then potentially complex with a second (reduced) 1,2,3-dithiazole/1,2,3-thiaselenazole core to form a stable complex and supported by the knowledge that selenium is also able coordinate bivalent zinc cations.[46-47] This mechanism is is analogous to work previously reported on the epidithiodiketopiperazine class of natural products.[45,48-49]

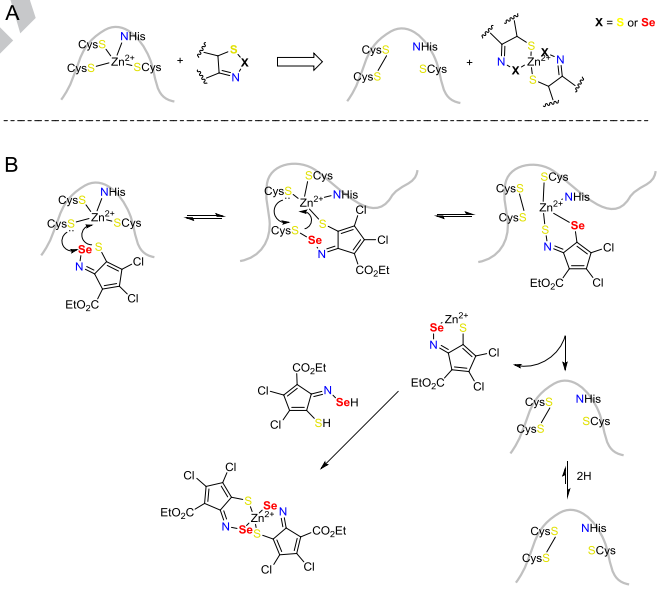


Figure 3. Proposed mechanism of action of 1,2,3-dithiazole/1,2,3-thiaselenazole for modification of NCp ***A*** - summary of the mechanism of zinc ejection from NCp/NCp7. ***B*** - a Zn^{2+}-coordinating cysteine thiol(ate) reacts with the disulfide of the 1,2,3-dithiazole/1,2,3-thiaselenazole core to generate a transient disulfide. The disulfide then rearranges to form an intramolecular protein disulfide with consequent reduction in zinc ion affinity. The ejected zinc ion (or part Zn^{2+} complex) can then complex with a second 1,2,3-dithiazole/1,2,3-thiaselenazole core (reduced) to form a stable complex.

The nanomolar potencies of compounds described in this study together with their corresponding lower toxicities show a progression towards the development of a useful candidate compound for the targeting of the FIV/HIV nucleocapsid protein. The structure activity relationship insights gained in the work particularly relating to reducing toxicity in **13** while maintaining potency in **15** demonstrates that this is a tractable target.

Compounds **9a** and **11c** highlight the benefits of employing selenium as a substitute for sulfur, producing a boost in anti-viral efficacy with a toxicity window comparable to the matched paired 1,2,3-dithiazole. The nucleocapsid protein of FIV is a viable therapeutic target and presents an exciting opportunity for therapeutic development. This under-utilized higher-order animal model provides many advantages over other non-primate alternatives. This is the first biological evaluation of a 1,2,3-thiaselenazole and with only two literature reports, this new class of compounds has exciting therapeutic potential

Acknowledgments

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