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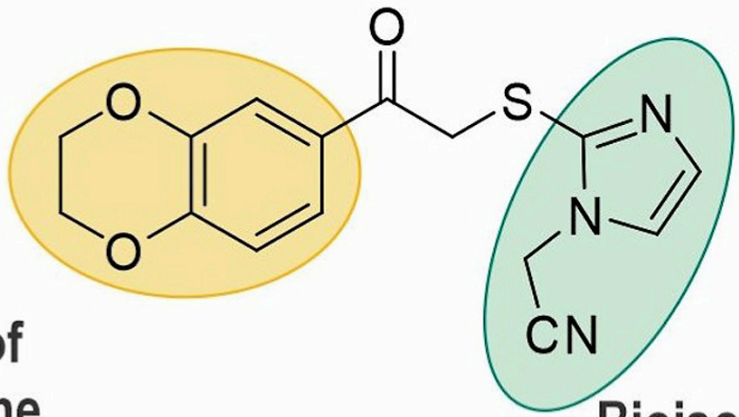
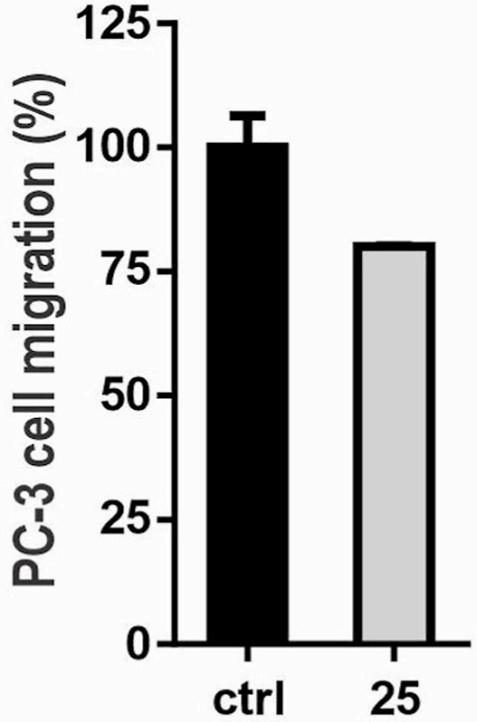
Variations of central linker



Variations of benzodioxine

25

Bioisosteric replacements of triazole



Structure-activity relationships of triazole-benzodioxine inhibitors of cathepsin X

Urša Pečar Fonović[a,*], Damijan Knez[a], Martina Hrast[a], Nace Zidar[a], Matic Proj[a], Stanislav Gobec[a], Janko Kos[a,b]

[a] University of Ljubljana, Faculty of Pharmacy, Aškerčeva 7, 1000 Ljubljana, Slovenia

[b] Department of Biotechnology, Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia

*Corresponding author:
Faculty of Pharmacy
University of Ljubljana
Aškerčeva 7
1000 Ljubljana, Slovenia
Tel: +386-1-4769-500
Email: ursa.pecarfonovic@ffa.uni-lj.si

Abstract

Cathepsin X is a cysteine carboxypeptidase that is involved in various physiological and pathological processes. In particular, highly elevated expression and activity of cathepsin X has been observed in cancers and neurodegenerative diseases. Previously, we identified compound **Z9** (1-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-((4-isopropyl-4*H*-1,2,4-triazol-3-yl)thio)ethan-1-one) as a potent and specific reversible cathepsin X inhibitor. Here, we have explored the effects of chemical variations to **Z9** of either benzodioxine or triazol moieties, and the importance of the central ketomethylenethio linker. The ketomethylenethio linker was crucial for cathepsin X inhibition, whereas changes of the triazole heterocycle did not alter the inhibitory potencies to a greater extent. Replacement of benzodioxine moiety with substituted benzenes reduced cathepsin X inhibition. Overall, several synthesized compounds showed similar or improved inhibitory potencies against cathepsin X compared to **Z9**, with IC_{50} values of 7.1 µM to 13.6 µM. Additionally, **25** inhibited prostate cancer cell migration by 21%, which is under the control of cathepsin X.

Keywords: cathepsin X; triazole-benzodioxine inhibitors; 1,2,4-triazole; anticancer drugs

Introduction

Cathepsin X (CatX) is a lysosomal carboxypeptidase that is expressed predominantly in immune and neuronal cells [1]. By cleaving diverse substrates, CatX can affect a number of different physiological processes, including cell proliferation, migration and adhesion [2]. In addition, it has also been implicated in various pathological processes, and particularly in cancers and neurodegenerative diseases. CatX is highly up-regulated in many cancers, where its proteolytic activity has a substantial role in the cleavage of different targets, such as profilin 1 and the β2-chain of integrin receptors, which are involved in cytoskeleton modulation and cell adhesion [3, 4]. Higher protein levels of CatX are associated with cancer progression and have been related to poor survival of cancer patients [5, 6]. As CatX activity cannot be regulated by endogenous cysteine peptidase inhibitors, it represents a promising target for the development of novel anticancer drugs [7]. Up-regulation of CatX has also been detected in several neurodegenerative diseases, including Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis, and Huntington's disease (for review, see Pišlar and Kos [8]). CatX is involved in the microglia-activation-mediated neurodegeneration [9] that abolishes the neuroprotective activity of γ-enolase [10]. As for cancers, inhibition of CatX activity during microglia activation might be a target to prevent and treat neurodegenerative disorders [9].

AMS-36 was the first irreversible epoxysuccinyl-based inhibitor of the papain family of cysteine proteases that showed some degree of specificity towards CatX in mice tumors [11]. Later, we identified several triazole-based compounds that can act as selective CatX inhibitors, and we revealed their reversible and competitive mechanism of action [12]. The most potent inhibitor was 1-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-((4-isopropyl-4*H*-1,2,4-triazol-3-yl)thio)ethan-1-one (Fig. 1, compound **Z9**), with *Ki* against CatX of 2.45 ± 0.05 µM. Compound **Z9** inhibited CatX by more than 75% in a kinetic assay using Abz-FEK(Dnp)-OH as substrate. **Z9** showed good selectivity in comparison to other related cysteine peptidases, and was not cytotoxic to PC-3 or PC-12 cells at concentrations up to 10 µM. Importantly, **Z9** significantly inhibited the migration of tumor cells and the outgrowth of neurites, which are two processes under CatX control [12].

We report herein a concise series of **Z9** variations to further explore the structure-activity relationships of the triazole-2,3-dihydrobenzo[*b*][1,4]dioxine class of inhibitors. Selected compounds showed improved inhibitory activities against CatX compared to **Z9**. The effects

of these new inhibitors were tested in cell-based assays, where compound **25** significantly reduced the migration of PC-3 cells *in vitro*.

Results and Discussion

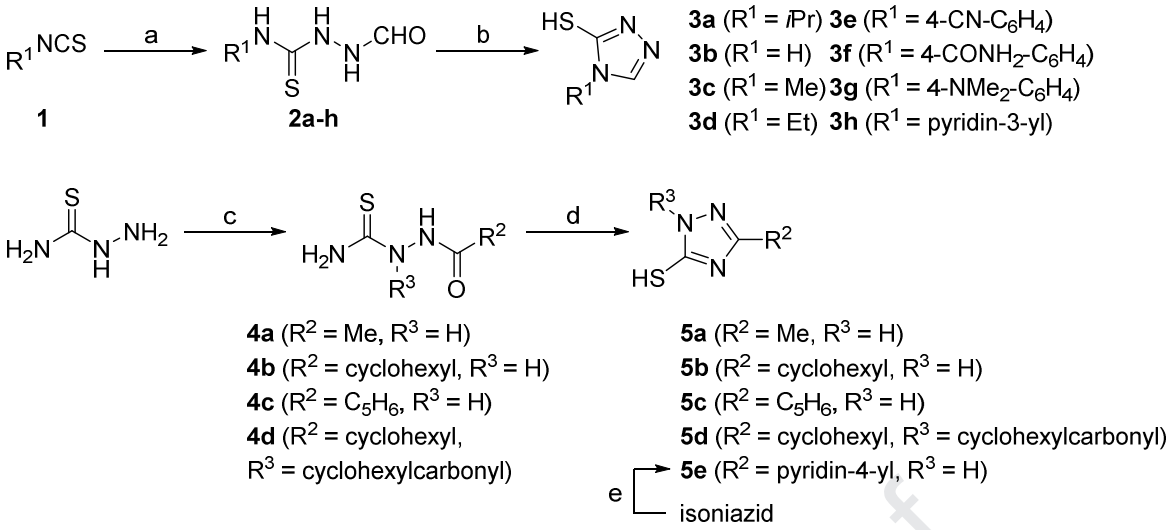
Design

The low molecular weight of the parent compound **Z9** and its simple modular structure allowed numerous structural modifications to explore the chemical space and assess the importance of the fragments for the inhibition of CatX. In this design, three key modifications to **Z9** were pursued (Fig. 1).

First, several different heterocyclic rings were introduced to replace the 4-isopropyl-4*H*-1,2,4-triazole-3-thio fragment. We established previously that this part of the triazole-2,3-dihydrobenzo[*b*][1,4]dioxine-based CatX inhibitors allows major changes, which might enhance the inhibitory potency [12]. We then investigated the importance of the ketomethylenethio linker structure by changing the sulfur group for an oxygen or amino group. The keto group was also transformed into secondary amino, hydroxyl, and methoxyimino groups. By preparing ketone replacements, we wanted to examine whether this fragment forms a hydrogen bond with the backbone NH of Gly73 of CatX, as indicated by molecular modeling studies of **Z9** in the procathepsin X with deleted proregion (PDB: 1DEU) [12]. Finally, we prepared a variety of compounds with diverse substituents that replaced the 1,4-benzodioxan-6-yl moiety, which does not form any specific interactions in the S2 pocket of CatX. The sequestering of additional interactions would potentially improve not only the potency, but also the selectivity of inhibitors of CatX, compared to other structurally similar cathepsin peptidases.

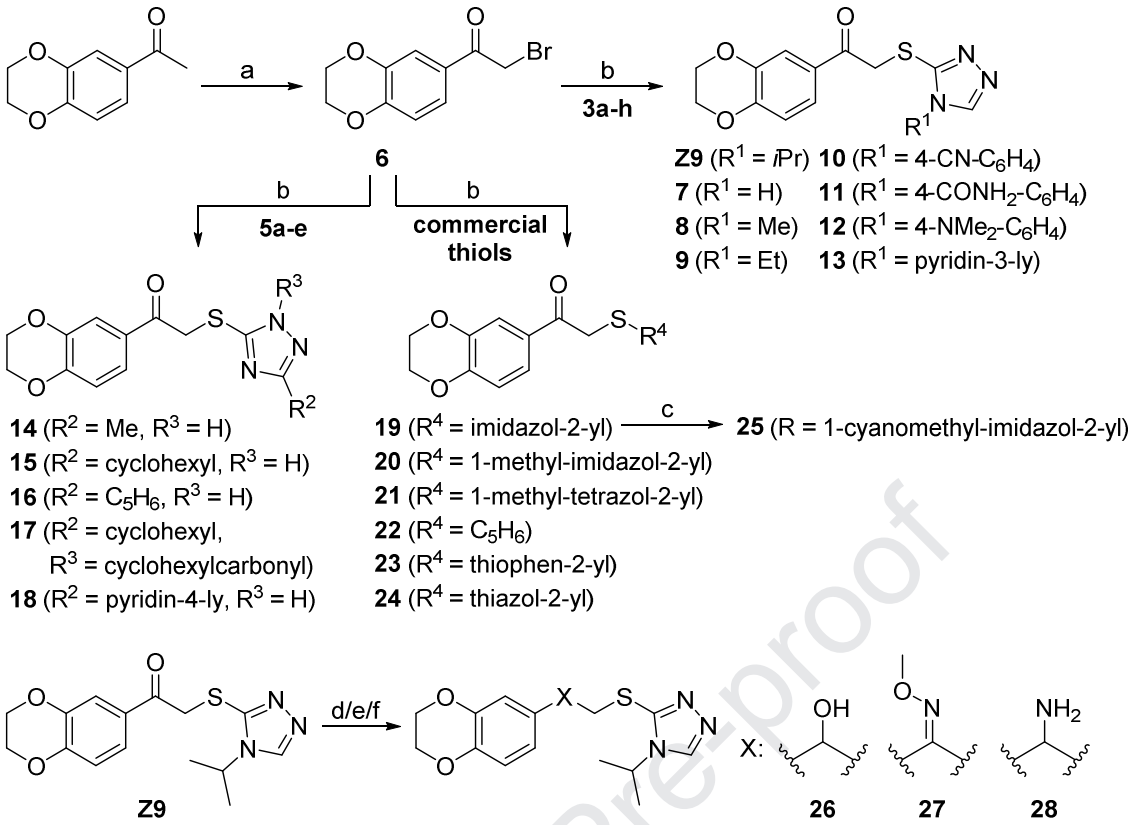
Chemistry

Analogues of **Z9** were synthesized according to Schemes 1-4. First, a small library of 1,2,4-triazole-3-thiols (**3a-h**) and 1,2,4-triazole-5-thiols (**5a-e**) was prepared (Scheme 1). For the synthesis of **3a-h**, different isothiocyanates (**1**) were reacted with formylhydrazine to give thiosemicarbazides **2a-h**, which then underwent a cyclodehydration under basic conditions to produce **3a-h** [13]. For the synthesis of **5a-d**, the reaction between different acyl chlorides and thiosemicarbazide gave acylthiosemicarbazides **4a-c**, which were then cyclized in alkaline medium to yield their corresponding 1,2,4-triazole-5-thiols 5**a-d**. Compound **5e** was prepared in a reaction between isoniazid and ammonium thiocyanate in refluxing 1 M HCl.



Scheme 1. Reagents and conditions: (a) NH_2NHCHO, EtOH, 90 °C, 18 h; (b) NaOH, H_2O, EtOH, 90 °C, 2 h; (c) corresponding acid chloride or acid, various conditions; (d) 2 M NaOH(aq), reflux 4 h; (e) NH_4SCN, 1 M HCl, reflux, 18 h.

In the next step, commercially available 1,4-benzodioxan-6-yl methyl ketone was selectively α-brominated with copper(II) bromide to yield α-bromo ketone **6**, with no aromatic ring bromination or dibromination of the α-methylene group, which are frequent side reactions of bromination (Scheme 2). Compound **6** was then reacted with thiols **3a-h**, **5a-e** or with various commercially available thiols in anhydrous ethanol to provide the desired products **Z9**, **10**–**24**. Compound **7a** (Table S1) was obtained as the side product in the synthesis of **7**, where the nucleophilic *N1*-nitrogen of the 1,2,4-triazole reacted with the carbonyl group, followed by elimination of water, to provide the bicyclic thiazolo[3,2-*b*][1,2,4]triazole system [14]. Compound **25** was prepared from **19** by alkylating the *N1*-nitrogen with chloroacetonitrile in the presence of potassium carbonate as base. The carbonyl group of **Z9** was transformed into an alcohol (**26**), an *O*-methyl oxime (**27**) or a secondary amine (**28**), using established procedures, as shown in Scheme 2.

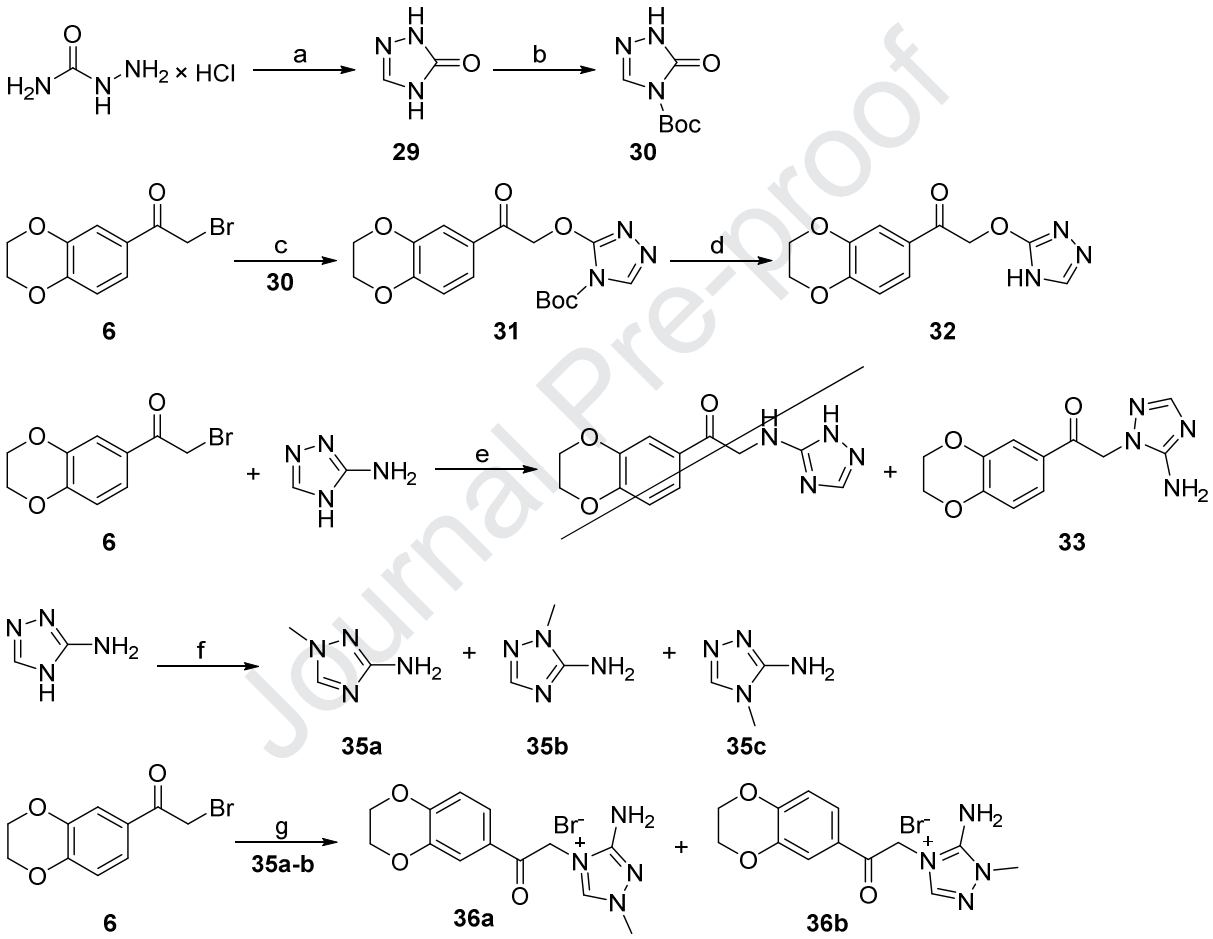


Scheme 2. Reagents and conditions: (a) $CuBr_2$, $CHCl_3$, 70 °C, 20 h; (b) anh EtOH, 90 °C, 20 h; (c) chloroacetonitrile, K_2CO_3, MeCN, reflux, 18 h; (d) $NaBH_4$, EtOH/THF (1/1), rt, 2 h (to obtain **26**); (e) *O*-methylhydroxylamine hydrochloride, pyridine, anh EtOH, 90 °C, 16 h (to obtain **27**); (f) NH_4OAc, $NaBH_3CN$, MeOH, 70 °C, 40 h (to obtain **28**).

Analog **32**, in which the thioether group in the linker is bioisosterically replaced with the ether group, was prepared according to Scheme 3. First, 1,2,4-triazolin-5-one (**29**) was synthesized from semicarbazide hydrochloride and trimethyl orthoformate, followed by protection of *N4*-nitrogen with the Boc protecting group (**30**). Then, NaH was added to **30** to form the enolate ion, which was alkylated with α-bromoketone **6** to yield **31**. Finally, the desired **32** was obtained by Boc-deprotection of **31** under anhydrous acidic conditions.

Replacement of the thioether group in the linker with the amino group was also attempted. By reacting the unsubstituted 4*H*-1,2,4-triazol-3-amine with α-bromoketone **6**, we did not obtain the desired compound with 3-amino group substitution, alternatively, only the *N1*-alkylated product **33** was obtained. To limit the possibility of alkylation of the ring nitrogen, 1,2,4-triazol-3-amines with monomethylated ring nitrogens were prepared first, according to the literature procedure [15]. According to this procedure, intermediates with *N*,*N*-dimethylformimidamide group are produced first (**34a-c**, not shown in Scheme 3), which are then hydrolyzed to methyl-1,2,4-triazol-3-amines. 1-Methyl-1*H*-1,2,4-triazol-3-amine

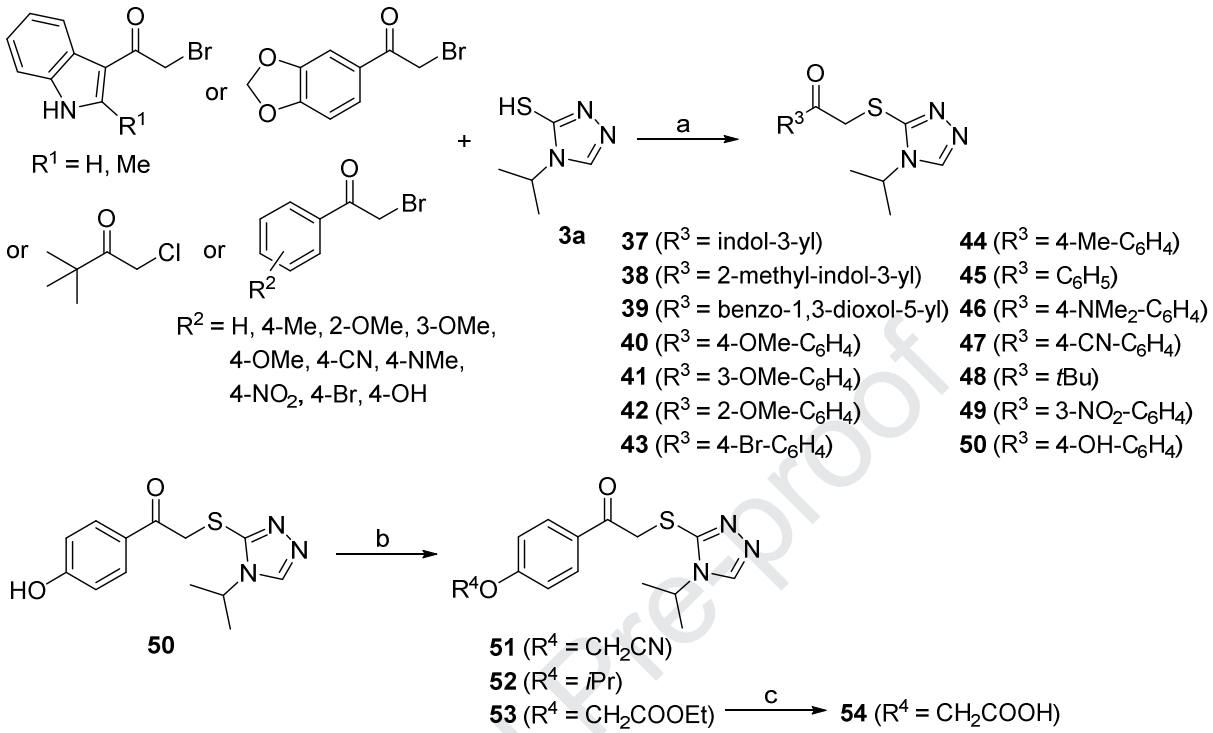
(**35a**) and 1-methyl-1*H*-1,2,4-triazol-5-amine (**35b**) were successfully isolated, whereas 4-methyl-4*H*-1,2,4-triazol-3-amine (**35c**) was not produced in sufficient quantity to proceed with the next reaction step. The isolated monomethylated 1,2,4-triazoles **35a** and **35b** were reacted with α-bromoketone **6** in anhydrous EtOH under reflux. Despite introducing a methyl group on the heterocyclic nitrogen, the primary aromatic amino group was not sufficiently nucleophilic, and only the quaternary ammonium derivatives **36a** and **36b** were obtained (Scheme 3).



Scheme 3. Reagents and conditions: (a) trimethyl orthoformate, MeOH, rt, 2 h; (b) $(BOC)_2O$, DMAP, DIPEA, DCM, rt, 18 h; (c) 1. **30**, NaH, anh DMF, rt, 15 min; 2. **6**, rt, 18 h; (d) TFA, DCM, rt, 2 h; (e) K_2CO_3, anh EtOH, rt, 18 h; (f) 1. DMFDMA, 110 °C, 18 h; 2. 4 M $NaOH_{(aq)}$, 110 °C, 1 h; (g) anh EtOH, 90 °C, 20 h.

Finally, a variety of compounds with diverse replacements of the 1,4-benzodioxan-6-yl fragment were prepared by reacting commercially available α-haloketones with 4-isopropyl-4*H*-1,2,4-triazole-3-thiol (**3a**) (Scheme 4). The 4-hydroxyl group in **50** was then

alkylated with small substituents to probe the chemical space (**51**–**53**). Compound **54** was prepared from **53** by alkaline hydrolysis of ethyl ester group.



Scheme 4. Reagents and conditions: (a) anh EtOH, 90 °C, 20 h; (b) corresponding alkyl halogenide, K_2CO_3, MeCN, 50 °C, 18 h; (c) 0.1 M $NaOH_{(aq)}$, MeOH, rt, 4 h.

Relative inhibition of cathepsin X and structure-activity relationships

Based on **Z9** [12], 45 new analogs were designed and prepared. These compounds were screened for their relative inhibition of CatX at 50 µM, using Abz-FEK(Dnp)-OH as the specific CatX substrate [16]. Several of these compounds inhibited CatX by >75% (Tables 1, 2).

Based on the data presented in Tables 1 and 2 and in Table S1, the structural features necessary for CatX inhibition can be deduced, and are summarized in Fig. 2. A wide variety of substituents on position 4 of the 4*H*-1,2,4-triazole-3-yl ring in compounds **7–13** (i.e., hydrogen, methyl, ethyl, isopropyl, *p*-cyanophenyl, pyridine-3-yl) did not significantly alter the inhibition, compared to **Z9**. The same was observed in our previous study, where diversely substituted triazoles **1**, **2**, **12**, **14**, **17**, **20** and **22** – compound **Z9** (numbering of compounds from reference [12]) displayed comparable inhibitory potencies. Similarly, smaller (i.e, methyl, **14**) or larger (e.g., phenyl, **16**) fragments on position 3 of triazole did not

have significant impact on the inhibitory profile of these compounds (Table 1, residual activities from 25% to 33%). Bioisosteric replacement of 4*H*-1,2,4-triazole (**7**) with imidazole (**19**), 1-methyl-1*H*-tetrazole (**21**), and thiazole (**24**) resulted in active compounds *versus* CatX, whereas introduction of phenyl (**22**) or thiophene (**23**) resulted in slightly decreased inhibition of CatX. The presence of a nitrogen atom in the heterocycle thus appears to enhance the inhibitory activity of these compounds. Transformation of the ketone to hydroxyl (**26**), amino (**28**), and *O*-methylhydroxylamine (**27**) completely abolished the inhibitory potency. Also, changing of the thioether group in the linker for an ether group resulted in an inactive compound (**32**; residual activity, 71% at 50 μM). The preparation of compounds with an amino group in the linker was also attempted, but as indicated above, only derivatives with substituted ring nitrogens were obtained (Table S1). These derivatives were not active against CatX, and together they show the pivotal role of the ketomethylenethio linker for potent CatX inhibition. The fragments that comprise the **Z9** molecule were also tested, but neither 1-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)ethan-1-one (**6ACBD**), bromide (**6**), alcohol (**55**) nor 4-isopropyl-4*H*-1,2,4-triazole-3-thiol (**3a**) alone were sufficient to inhibit CatX (Table S1).

Next, changing the left-hand side of the molecule (Scheme 4) to bicyclic systems such as indole (**37**) and benzo[*d*][1,3]dioxole (**39**) retained relative inhibitions <50% (Table 2). However, substituted phenyl derivatives (**40**-**54**) had significantly lower inhibitory activities in comparison to **Z9** (Table 2).

To rationalize the structure-activity relationships described above, molecular modeling was performed. The modeling studies using ligand-free crystal structure of CatX (PDB: 1EF7) suggested that compounds studied bind into the active site, where the best scores and superpositions of ligands were achieved (Supplementary information, Fig. S1). Docking studies did not indicate binding into potential allosteric binding sites as was seen in the case of cathepsin K (PDB: 6ASH, 5JA7, 5J94). Keto group of the ketomethylenethio linker forms a hydrogen bond with Gln22 side chain, which stabilizes inhibitors in the active site groove, and explains why modifications of keto group resulted in inactive compounds. Moreover, additional H-bonds between the Gly74 and Asn179, and nitrogens of the triazole and its bioisosteres enhance inhibitory potencies, whereas phenyl (**23**) and thiophene (**24**) analogues are inactive. The modelling studies did not show any specific interactions for the 1,4-benzodioxan-6-yl moiety. In general, phenyl analogues (**40**–**45**) had higher docking score, which is also in correlation with lower inhibitory activity obtained by the biochemical evaluation.

The IC_{50} values were further determined for five compounds that demonstrated improved or similar inhibitory potencies to the parent **Z9** (Table 3). All of these derivatives showed IC_{50} values in the low micromolar range, which were comparable to **Z9**. The dilution assay confirmed the reversible binding of the compounds (Supplementary information, Fig. S2). Compound **25** with cyanomethylene residue, which could form a covalent thioimidate adduct with catalytic Cys31 [17], is a non-time dependent inhibitor of CatX (Supplementary information, Fig. S3). The progress curves were identical as reported in our previous communication, which indicates instantaneous competitive inhibition with preceding slow inhibitor isomerization [12]. Moreover, inhibitory potencies towards cathepsins B (endo- and exo-peptidase activities), L and S were determined. At 100 μM, no or lower than 30% inhibition of the cathepsins' activity was observed. The IC_{50} value of compound **25** against cathepsin S was determined (241 ± 26.9 μM). These results show good selectivity profile of studied compounds for CatX.

Cytotoxicity of compounds and their effects on migration of PC-3 cells

To distinguish the effects in migration assays that were caused by specific mechanisms from those due to triggering of cell cytotoxicity, the cytotoxic effects of the selected compounds were initially investigated for PC-3 cells, to select concentrations that did not decrease cell viability. At 10 μM, **10** and **20** showed weak cytotoxic effects after 24 h and 48 h, while **10** was the only compound that showed statistically significant cytotoxicity at 20 μM, at both 24 h and 48 h. The other compounds tested did not affect cell viability after 24 h or 48 h (Fig. 3). As CatX is involved in cell migration, we investigated how these compounds impeded this process. **Z9** was used as the positive control, as we have already shown that it has inhibitory effects on PC-3 cell migration. Of the two compounds tested, **25** decreased cell migration by 21%, but at twice the concentration of **Z9**, while **8** had no effects (Fig. 4). The lower effect of **25** on cell migration in comparison with **Z9** could be a result of lower permeation of cell membrane of the former as also predicted by the QikProp prediction software (Supplementary information, Table S2; Schrödinger Release 2019-3, Schrödinger, LLC, NY, 2019).

Conclusions

In summary, an extensive series of CatX inhibitors was developed based on the 1,2,4-triazole parent compound **Z9**. Several of these compounds showed inhibitory activities against CatX

in the same concentration range as **Z9**, with IC_{50} values from 7.13 µM to 13.6 µM, thereby providing important information about their structure-activity relationships. Compound **25**, the most potent of these CatX inhibitors, was not cytotoxic to PC-3 cells at ≤20 µM, and decreased PC-3 cell migration. These data show that a possible approach for further improvement of the inhibitory potency lies in the modification of the triazole substituents, and highlight the therapeutic antitumor potential of this class of CatX inhibitors.

Experimental Methods

General synthetic chemistry experimental protocols

The reagents and solvents used were obtained from commercial sources (i.e., Acros Organics, Sigma Aldrich, TCI Europe, Merck, Carlo Erba, Apollo Scientific, Alfa Aesar) and were used as provided, unless otherwise indicated. Analytical thin-layer chromatography was performed on silica gel aluminium sheets (60 F254, 0.20 mm; Merck), with visualization using UV light (254 nm) and/or visualization reagents (i.e., ninhydrine, 2,4-dinitrophenylhydrazine). Flash column chromatography was performed on silica gel 60 (particle size, 0.040–0.063 mm; 230–400 mesh; Merck). ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 MHz and 100 MHz, respectively, on a nuclear magnetic resonance (NMR) spectrometer (Avance III; Bruker Corporation, MA, USA), at 295 K. The chemical shifts (δ) are reported in parts per million (ppm) and are referenced to the deuterated solvent used. High-resolution mass spectrometry (HRMS) measurements were performed (Autospec Q Micromass mass spectrometer; Fisons, VG Analytical, Manchester, UK) at the Jozef Stefan Institute, Ljubljana, Slovenia, and on a liquid chromatography (LC)–tandem mass spectrometry (MS/MS) system (Q Executive Plus; Thermo Scientific, MA, USA). The MS measurements were performed on a mass spectrometer (Expression CMS; Advion, NY, USA). Analytical reversed-phase HPLC analysis was performed on a modular system (Dionex UltiMate 3000; Thermo Fisher Scientific Inc., MA, USA).

General method: C18 column (Acquity UPLC HSS; 1.8 µm, 2.1 × 50 mm; Waters), T = 40 °C; injection volume 1 µL, sample 0.2 mg/mL (4% DMSO in MeCN); flow rate, 0.4 mL/min; detector λ = 254 nm; mobile phase A (0.1% TFA [v/v] in water), mobile phase B (MeCN). Gradient (for mobile phase B): 0–7 min, 10%–90%; 7–10 min, 90%. Purities of the tested compounds were established as ≥95% by HPLC, unless stated otherwise.

Synthesis

General procedure for synthesis of thiosemicarbazides (**2a-h**): To a suspension of formylhydrazine (2.0 g, 33.5 mmol, 1.0 equiv.) in EtOH (100 mL), the corresponding isothiocyanate (1.0 equiv.) was added. The reaction mixture was stirred at 90 °C for 20 h. The volume of EtOH was reduced to half by evaporation under reduced pressure, and then the reaction mixture was cooled to room temperature. The white precipitate was filtered off to obtain the crude product, which was used in the next step without purification. As estimated by NMR, the thiosemicarbazides were already partially cyclized to their corresponding 5-mercapto-4-substituted-1,2,4-triazoles.

General procedure for synthesis of 1,2,4-triazole-3-thiols (**3a-h**): To a suspension of thiosemicarbazides **2a-h** (10 mmol, 1.0 equiv.) in EtOH, 4 M NaOH(aq) (5 mL, 1.5 equiv.) was added, and the reaction mixture was refluxed at 90 °C for 4 h. The mixture was cooled to room temperature, acidified with 2 M HCl(aq) to pH ~5, and the EtOH was evaporated off under reduced pressure. The precipitate that formed was filtered off, yielding the pure product, which was used in the next step.

4-Isopropyl-4*H*-1,2,4-triazole-3-thiol (**3a**). Yield, 80%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.34 (d, J = 6.9 Hz, 6H), 4.63 (hept, J = 6.8 Hz, 1 H), 8.60 (s, 1 H), 13.70 (bs, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 21.37, 46.88, 139.53, 164.97 ppm. ESI-MS for $C_5H_8N_3S$ $[M–H]^-$ 142.11. HRMS (ESI) m/z calculated for $C_5H_{10}O_3S$ $[M + H]^+$ 144.0590, found 144.0587.

4*H*-1,2,4-Triazole-3-thiol (**3b**). Commercially available reagent was used.

4-Methyl-4*H*-1,2,4-triazole-3-thiol (**3c**). Yield, 25%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.43 (s, 3 H), 8.40 (s, 1 H), 13.65 (bs, 1 H) ppm. ESI-MS for $C_3H_4N_3S$ $[M – H]^-$ 144.13.

4-Ethyl-4*H*-1,2,4-triazole-3-thiol (**3d**). Yield, 60%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.27 (t, J = 7.3 Hz, 3 H), 3.92 (q, J = 7.3 Hz, 2 H), 8.47 (d, J = 1.6 Hz, 1 H), 13.67 (bs, 1 H) ppm. ESI-MS for $C_4H_8N_3S$+MeOH $[M+MeOH+H]^+$ 161.97.

4-(3-Mercapto-4*H*-1,2,4-triazol-4-yl)benzonitrile (**3e**). Yield, 77%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.98 (d, J = 8.7 Hz, 2 H), 8.08 (d, J = 8.7 Hz, 2 H), 8.81 (d, J = 1.6 Hz, 1 H), 14.09 (bs, 1 H) ppm.

4-(3-Mercapto-4*H*-1,2,4-triazol-4-yl)benzamide (**3f**). Yield, 23%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.54 (bs, 1 H), 7.78 (d, J = 8.6 Hz, 2 H), 8.02 (d, J = 8.6 Hz, 2 H), 8.12 (bs, 1 H), 8.77 (d, J = 1.6 Hz, 1 H), 14.01 (bs, 1H) ppm.

4-(4-(Dimethylamino)phenyl)-4*H*-1,2,4-triazole-3-thiol (**3g**). Yield, 91%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.95 (s, 6 H), 6.81 (d, J = 9.1 Hz, 2 H), 7.37 (d, J = 9.1 Hz, 2 H), 8.57 (d, J = 1.6 Hz, 1 H), 13.82 (bs, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 39.93 (under DMSO peak, confirmed by 2D NMR experiment ^{1}H-^{13}C HSQC), 111.86, 122.68, 126.49, 142.41, 150.27, 166.39 ppm. ESI-MS for $C_{10}H_{13}N_4S$ $[M+H]^+$ 221.05.

4-(Pyridin-3-yl)-4*H*-1,2,4-triazole-3-thiol (**3h**). Yield, 73%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.62 (ddd, J = 8.2, 4.8, 0.8 Hz, 1 H), 8.16 (ddd, J = 8.2, 2.6, 1.5 Hz, 1 H), 8.68 (dd, J = 4.8, 1.5 Hz, 1 H), 8.79 (s, 1 H), 8.86 (dd, J = 2.6, 0.7 Hz, 1 H), 14.07 (bs, 1 H) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ 123.87, 131.15, 133.89, 142.07, 146.71, 149.70, 166.55 ppm. ESI-MS for $C_7H_7N_4S$ $[M + H]^+$ 178.91.

Synthesis of 2-acetylhydrazine-1-carbothioamide (**4a**): Acetic acid (20 mL) was added to thiosemicarbazide (4,55 g, 50 mmol, 1.0 equiv.), and the mixture was stirred at reflux (130 °C) for 4 h. The reaction was cooled to room temperature, the solvent was evaporated under reduced pressure, and the crude product was recrystallized from water. Yield, 65%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.82 (s, 3 H), 7,48 (s, 1 H), 7.83 (s, 1 H), 9.15 (s, 1 H), 9.70 (s, 1 H) ppm.

Synthesis of 2-(cyclohexanecarbonyl)hydrazine-1-carbothioamide (**4b**): To a solution of cyclohexyl carboxylic acid (3.0 g, 23.4 mmol, 1.0 equiv) in DCM (20 mL), oxalyl chloride (6.0 mL, 70.2 mmol, 3.0 equiv.) and a catalytic amount of DMF were added, and the reaction mixture was stirred at 45 °C for 1 h. The mixture was cooled to room temperature, and the solvent was evaporated under reduced pressure. The residue was dissolved in anhydrous THF (20 mL), followed by addition of thiosemicarbazide (4.26 g, 46.8 mmol, 2 equiv.) and pyridine (3.0 mL, 35.1 mmol, 1.5 equiv.). The mixture was stirred at room temperature for 18 h. EtOAc (100 mL) was then added and the organic phase was washed with water (2 × 50 mL) and brine (50 mL), and then dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the product was purified by flash chromatography using DCM/MeOH (20:1, v/v) as mobile phase. The crude product was used in the next step without further purification.

Synthesis of 2-benzoylhydrazine-1-carbothioamide (**4c**). To a cold solution (0 °C) of benzoyl chloride (1.17 mL, 10.0 mmol, 1.0 equiv.) in THF (15 mL), thiosemicarbazide (1.0 g, 10.9 mmol, 1.1 equiv.) was added in aliquots, and the reaction was stirred at room temperature for 6 h. The solvent was evaporated under reduced pressure, the residue was dissolved in EtOAc (30 mL), and the organic phase was washed with saturated $NaHCO_3$(aq) (2 × 15 mL), then dried over Na_2SO_4, filtered and evaporated. The product was recrystallized from EtOH and used without further purification. Yield, 34%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.47–7.50 (m, 2 H), 7.55–7.59 (m, 1 H), 7.65 (bs, 1 H), 7.89 – 7.92 (m, 3 H), 9.36 (s, 1 H), 10.40 (s, 1 H) ppm. ESI-MS for $C_8H_{10}N_3OS$ $[M + H]^+$ 196.02 [18].

General procedure for cyclization of carbothioamides (**5a-c**): A suspension of acylthiosemicarbazides **4a-c** (2.5 mmol, 1.0 equiv.) in 2 M NaOH(aq) (2 mL, 4 mmol, 1.6 equiv.) was stirred at reflux for 4 h. The mixture was cooled to room temperature and acidified with concentrated HCl to pH ~3. The precipitate formed was filtered off, recrystallized from MeOH, and used without further purification.

5-Methyl-4*H*-1,2,4-triazole-3-thiol (**5a**). Yield, 58%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.16 (s, 3 H), 13.05 (bs, 1 H), 13.15 (s, 1 H) ppm. ESI-MS for $C_3H_6N_3S$ $[M + H]^+$ 116.02.

3-Cyclohexyl-1*H*-1,2,4-triazole-5-thiol (**5b**). Crude intermediate **4b** was used as starting compound. Yield, 44%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.13–1.46 (m, 5 H), 1.60–1.65 (m, 1 H), 1.71 (dt, J = 12.5, 3.5 Hz, 2 H), 1.84–1.88 (m, 2 H), 2.56 (tt, J = 11.3, 3.5 Hz, 1 H), 13.07 (s, 1 H), 13.19 (s, 1 H) ppm. ESI-MS for $C_8H_{14}N_3S$ $[M + H]^+$ 183.99.

5-Phenyl-4*H*-1,2,4-triazole-3-thiol (**5c**). Yield, 61%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.50–7.53 (m. 3 H), 7.89–7.92 (m, 2 H), 13.69 (s, 1 H), 13.86 (s, 1 H) ppm. ESI-MS for $C_8H_8N_3S$ $[M + H]^+$ 177.96 [19].

Synthesis of cyclohexyl(3-cyclohexyl-5-mercapto-1H-1,2,4-triazol-1-yl)methanone (**5d**): To a solution of cyclohexyl carboxylic acid (3.0 g, 23.4 mmol, 1.0 equiv.) in DCM (20 mL), oxalyl chloride (6.0 mL, 70.2 mmol, 3.0 equiv.) and a catalytic amount of DMF were added and stirred at 45 °C for 1 h. The mixture was cooled to room temperature and the solvent was evaporated under reduced pressure. The residue was dissolved in DCM (20 mL), followed by addition of thiosemicarbazide (2.13 g, 23.4 mmol, 1 equiv.) and pyridine (3 mL, 35.1 mmol, 1.5 equiv.). The mixture was stirred at room temperature for 18 h to yield intermediate **4d**

(1,2-di(cyclohexanecarbonyl)hydrazine-1-carbothioamide) The solvent was evaporated under reduced pressure, and 2 M NaOH(aq) (5 mL) was added to the crude intermediate **4d**, and stirred at reflux for 4 h. The reaction was cooled to room temperature, the solvent was evaporated off under reduced pressure, and the product was isolated by flash chromatography using DCM/MeOH (20:1, v/v) as mobile phase. Yield, 3%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.28–1.38 (m, 2 H), 1.42–1.51 (m, 4 H), 1.55–1.67 (m, 4 H), 1.74–1.78 (m, 2 H), 1.83–1.91 (m, 4 H), 1.99–2.02 (m, 2 H), 2.14–2.18 (m, 2 H), 2.84–2.90 (m, 1 H), 3.04–3.10 (m, 1 H), 13.05 (s, 1 H) ppm. ESI-MS for $C_{15}H_{24}N_3OS$ $[M + H]^+$ 294.08.

Synthesis of 3-(pyridin-4-yl)-1H-1,2,4-triazole-5-thiol (**5e**): To a solution of isoniazid (1.371 g, 20.0 mmol, 1.0 equiv.) in 1 M HCl(aq) (5 mL), NH_4SCN (1.52 g, 20.0 mmol, 1.0 equiv.) was added, and the reaction was stirred at reflux for 18 h. The mixture was cooled to room temperature, and the forming yellow precipitate was filtered off, yielding the pure product. Yield, 45%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.84 (dd, J = 4.4, 1.6 Hz, 2 H), 8.73 (dd, J = 4.4, 2.0 Hz, 2 H), 13.94 (s, 1 H), 14.11 (bs, 1 H) ppm. ESI-MS for $C_7H_7N_4S$ $[M + H]^+$ 178.97.

Synthesis of 2-bromo-1-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)ethan-1-one (**6**). To a solution of 1-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)ethan-1-one (5.0 g, 28.0 mmol, 1.0 equiv.) in chloroform (100 mL), $CuBr_2$ (10.7 g, 47.7 mmol, 1.7 equiv.) was added in one aliquot. The reaction mixture was stirred at 70 °C for 20 h. After cooling the mixture to room temperature, the excess $CuBr_2$ and CuBr (formed as side product) were filtered off. The product was purified by flash chromatograph using DCM/Hex (4:1, v/v) as mobile phase. Yield, 67%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.27–4.29 (m, 2 H), 4.31–4.34 (m, 2 H), 4.37 (s, 2 H), 6.91–6.93 (m, 1 H), 7.49–7.52 (m, 2 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 30.89, 64.18, 64.85, 117.63, 118.53, 123.29, 127.73, 143.63, 148.91, 189.92 ppm. HRMS (ESI) m/z calculated for $C_{10}H_{10}O_3Br$ $[M + H]^+$ 256.9808, found 256.9799.

General procedure for synthesis of final products (**Z9**, **7-24**): The suspension of bromide **6** (8.0 mmol, 1.0 eq) and thiol (8.0 mmol, 1.0 equiv.) in anhydrous EtOH (30 mL) was stirred at 90 °C for 18 h. The reaction mixture was cooled to room temperature and the forming precipitate was filtered off, yielding the pure product. If necessary, the product was purified by flash chromatography.

1-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-((4-isopropyl-4*H*-1,2,4-triazol-3-yl)thio)ethan-1-one (**Z9**). Yield, 81%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.50 (d, J = 6.7 Hz, 6 H), 4.28–4.30

(m, 2 H), 4.33–4.35 (m, 2 H), 4.58 (hept, *J* = 6.7 Hz, 1 H), 5.04 (s, 2 H), 7.01 (d, *J* = 8.5 Hz, 1 H), 7.52 (d, *J* = 2.1 Hz, 1 H), 7.55 (dd, *J* = 8.5, 2.2 Hz, 1 H), 9.79 (s, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 21.87, 41.29, 50.29, 63.99, 64.69, 117.40, 117.58, 122.61, 128.38, 142.99, 143.30, 148.65, 150.62, 190.77 ppm. HRMS (ESI) m/z calculated for $C_{15}H_{18}N_3O_3S$ $[M + H]^+$ 320.1069, found 320.1066. HPLC purity, 99.3% at 254 nm (t_R = 3.57 min).

2-((4*H*-1,2,4-Triazol-3-yl)thio)-1-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)ethan-1-one (**7**). Yield, 23%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.28–4.30 (m, 2 H), 4.33–4.35 (m, 2 H), 4.68 (s, 2 H), 6.99 (d, *J* = 8.4 Hz, 1 H), 7.50 (d, *J* = 2.1 Hz, 1 H), 7.54 (dd, *J* = 8.4, 2.1 Hz, 1 H), 8.52 (s, 1H), 14.01 (s, 1H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 38.61, 63.97, 64.62, 117.23, 117.42, 122.43, 129.12, 143.22, 144.74, 148.19, 158.26, 192.15 ppm. HRMS (ESI) m/z calculated for $C_{12}H_{12}N_3O_3S$ $[M + H]^+$ 278.0599, found 278.0605. HPLC purity, 99.3% at 254 nm (t_R = 2.77 min).

6-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)thiazolo[3,2-*b*][1,2,4]triazole (**7a**, Supplementary information, Table S1). This compound was obtained as a side product in the synthesis of **7**. Yield, 37%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.31 (s, 4 H), 7.03 (d, *J* = 8.5 Hz, 1 H), 7.69 (dd, *J* = 8.5, 2.2 Hz, 1 H), 7.79 (d, *J* = 2.2 Hz, 1 H), 7.83 (d, *J* = 1.5 Hz, 1 H), 8.42 (d, *J* = 1.5 Hz, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 64.14, 64.35, 109.27, 115.06, 117.53, 119.57, 120.87, 131.07, 143.44, 144.64, 156.07, 156.67 ppm. ESI-MS for $C_{12}H_{10}N_3O_2S$ $[M + H]^+$ 260.16. HRMS (ESI) m/z calculated for $C_{12}H_{10}N_3O_2S$ $[M + H]^+$ 260.0488, found 260.0483. HPLC purity, 100% at 254 nm (t_R = 4.03 min).

1-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-((4-methyl-4*H*-1,2,4-triazol-3-yl)thio)ethan-1-one (**8**). Yield, 86%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.69 (s, 3 H), 4.28–4.31 (m, 2 H), 4.33–4.35 (m, 2 H), 4.89 (s, 2 H), 7.00 (d, *J* = 8.4 Hz, 1 H), 7.51 (d, *J* = 2.1 Hz, 1 H), 7.54 (dd, *J* = 8.4, 2.2 Hz, 1 H), 9.06 (s, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 31.79, 40.95, 63.96, 64.65, 117.32, 117.56, 122.56, 128.48, 143.25, 145.80, 148.52, 150.36, 191.22 ppm. HRMS (ESI) m/z calculated for $C_{13}H_{14}N_3O_3S$ $[M + H]^+$ 292.0756, found 292.0759. HPLC purity, 97.8% at 254 nm (t_R = 2.90 min).

1-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-((4-ethyl-4*H*-1,2,4-triazol-3-yl)thio)ethan-1-one (**9**). Yield, 40%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.42 (t, *J* = 7.3 Hz, 3 H), 4.14 (q, *J* = 7.3 Hz, 2 H), 4.28–4.30 (m, 2 H), 4.33–4.35 (m, 2 H), 5.02 (s, 2 H), 7.05 (d, *J* = 8.5 Hz, 1 H), 7.52 (d, *J* = 2.1 Hz, 1 H), 7.55 (dd, *J* = 8.4, 2.2 Hz, 1 H), 9.57 (s, 1H) ppm. ^{13}C NMR (100 MHz,

DMSO-d_6) δ 14.31, 41.26, 41.46, 64.00, 64.70, 117.40, 117.59, 122.61, 128.38, 143.30, 144.52, 148.64, 151.05, 190.80 ppm. HRMS (ESI) m/z calculated for $C_{14}H_{16}N_3O_3S$ $[M + H]^+$ 306.0907, found 306.0899. HPLC purity, 97.4% at 254 nm (t_R = 3.27 min).

4-(3-((2-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-oxoethyl)thio)-4*H*-1,2,4-triazol-4-yl)benzonitrile (**10**). Yield, 54%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.28–4.31 (m, 2 H), 4.33–4.35 (m, 2 H), 4.82 (s, 2 H), 6.98 (d, J = 8.4 Hz, 1 H), 7.45 (d, J = 2.1 Hz, 1 H), 7.51 (dd, J = 8.5, 2.2 Hz, 1 H), 7.81 (d, J = 8.6 Hz, 2 H), 8.10 (d, J = 8.7 Hz, 2 H), 8.96 (s, 1H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 40.68, 63.91, 64.58, 111.91, 117.19, 117.42, 117.95, 122.47, 126.10, 128.54, 133.94, 143.15, 145.13, 148.36, 148.36, 191.22 ppm. HRMS (ESI) m/z calculated for $C_{19}H_{14}N_4O_3S$ $[M + H]^+$ 379.0865, found 379.0865. HPLC purity, 95.4% at 254 nm (t_R = 3.93 min).

4-(3-((2-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-oxoethyl)thio)-4*H*-1,2,4-triazol-4-yl)benzamide (**11**). Yield, 14%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.29–4.31 (m, 2 H), 4.33–4.36 (m, 2 H), 4.85 (s, 2 H), 7.00 (d, J = 8.5 Hz, 1 H), 7.49 (d, J = 2.1 Hz, 1 H), 7.53 (dd, J = 8.5, 2.1 Hz, 1 H), 7.58 (bs, 1 H), 7.64 (d, J = 8.5 Hz, 2 H), 8.06 (d, J = 8.5 Hz, 2 H), 8.16 (bs, 1 H), 8.92 (s, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 40.40, 63.92, 64.59, 117.23, 117.43, 122.46, 124.93, 128.67, 128.94, 134.88, 135.46, 143.19, 145.18, 148.36, 148.60, 166.70, 191.27 ppm. HRMS (ESI) m/z calculated for $C_{19}H_{16}N_4O_4S$ $[M + H]^+$ 397.0971, found 397.0974. HPLC purity, 99.0% at 254 nm (t_R = 3.05 min).

1-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-((4-(4-(dimethylamino)phenyl)-4*H*-1,2,4-triazol-3-yl)thio)ethan-1-one (**12**). Yield, 65%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.98 (s, 6 H), 4.29–4.31 (m, 2 H), 4.33–4.35 (m, 2 H), 4.84 (s, 2 H), 6.86 (d, J = 9.0 Hz, 2 H), 7.00 (d, J = 8.5 Hz, 1 H), 7.31 (d, J = 9.0 Hz, 2 H), 7.50 (d, J = 2.0 Hz, 1 H), 7.54 (dd, J = 8.5, 2.0 Hz, 1 H), 8.90 (s, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 38.84, 40.10, 63.92, 64.59, 112.44, 117.24, 117.45, 121.33, 122.46, 126.12, 128.69, 143.19, 145.50, 148.37, 149.88, 150.60, 191.28 ppm. HRMS (ESI) m/z calculated for $C_{20}H_{20}N_4O_3S$ $[M + H]^+$ 397.1334, found 397.1329. HPLC purity, 98.2% at 254 nm (t_R = 3.97 min).

1-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-((4-(pyridin-3-yl)-4*H*-1,2,4-triazol-3-yl)thio)ethan-1-one (**13**). Yield, 25%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.29–4.31 (m, 2 H), 4.33–4.35 (m, 2 H), 4.82 (s, 2 H), 6.99 (d, J = 8.4 Hz, 1 H), 7.47 (d, J = 2.2 Hz, 1 H), 7.51 (dd, J = 8.4, 2.2 Hz, 1 H), 7.66 (dd, J = 8.4, 4.8 Hz, 1 H), 8.06 (ddd, J = 8.1, 2.6, 1.5 Hz, 1 H), 8.74

(dd, J = 8.4, 1.5 Hz, 1 H), 8.78 (d, J = 2.6 Hz, 1 H), 8.94 (s, 1H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 40.74, 63.92, 64.59, 117.22, 117.45, 122.47, 124.38, 128.57, 130.33, 133.39, 143.18, 145.37, 146.09, 148.37, 148.83, 150.28, 191.28 ppm. HRMS (ESI) m/z calculated for $C_{17}H_{14}N_4O_3S$ $[M + H]^+$ 355.0865, found 355.0862. HPLC purity, 97.2% at 254 nm (t_R = 3.32 min).

1-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-((5-methyl-4*H*-1,2,4-triazol-3-yl)thio)ethan-1-one (**14**). Yield, 79%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.45 (s, 3 H), 4.27–4.29 (m, 2 H), 4.32–4.34 (m, 2 H), 4.86 (s, 2 H), 6.99 (d, J = 8.4 Hz, 1 H), 7.49 (s, J = 2.1 Hz, 1 H), 7.53 (dd, J = 8.4, 2.1 Hz, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 10.99, 39.79 (under DMSO peak, confirmed by 2D NMR experiment ^{1}H-^{13}C HSQC), 64.01, 64.68, 117.32, 117.50, 122.54, 128.64, 143.26, 148.47, 153.78 (2 × Ar-C, confirmed by 2D NMR experiment ^{1}H-^{13}C HMBC), 191.28. HRMS (ESI) m/z calculated for $C_{13}H_{14}N_3O_3S$ $[M + H]^+$ 292.0750, found 292.0745. HPLC purity, 98.1% at 254 nm (t_R = 2.82 min).

2-((3-Cyclohexyl-1*H*-1,2,4-triazol-5-yl)thio)-1-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)ethan-1-one (**15**). Yield, 26%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.17–1.24 (m, 1 H), 1.27–1.48 (m, 4 H), 1.62–1.69 (m, 1 H), 1.70–1.75 (m, 2 H), 1.87–1.91 (m, 2 H), 2.67–2.75 (m, 1 H), 4.28–4.30 (m, 2 H), 4.32–4.35 (m, 2 H), 4.68 (s, 2 H), 6.99 (d, J = 8.3 Hz, 1 H), 7.50 (d, J = 2.1 Hz, 1 H), 7.53 (dd, J = 8.4, 2.1 Hz, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 25.09, 25.25, 30.58, 35.15, 38.61, 63.92, 64.57, 117.16, 117.44, 122.39, 129.01, 143.14, 148.18, 155.88, 161.81, 192.09 ppm. HRMS (ESI) m/z calculated for $C_{18}H_{22}N_3O_3S$ $[M + H]^+$ 360.1376, found 360.1373. HPLC purity, 98.5% at 254 nm (t_R = 4.08 min).

1-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-((5-phenyl-4*H*-1,2,4-triazol-3-yl)thio)ethan-1-one (**16**). Yield, 61%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.29–4.31 (m, 2 H), 4.33–4.35 (m, 2 H), 4.78 (s, 2 H), 7.01 (d, J = 8.4 Hz, 1 H), 7.46–7.50 (m, 3 H), 7.55–7.59 (m, 2 H), 7.89–7.91 (m, 2 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 38.91 (under DMSO peak, confirmed by 2D NMR experiment ^{1}H-^{13}C HSQC) 63.93, 64.58, 117.20, 117.50, 122.42, 125.88 (2 × Ar-C, confirmed 2D NMR experiment ^{1}H-^{13}C HSQC), 128.94 (3 × Ar-C, confirmed 2D NMR experiment ^{1}H-^{13}C HSQC), 126.00, 129.08, 129.93, 143.17, 148.21, 153.93, 192.14 ppm. HRMS (ESI) m/z calculated for $C_{18}H_{16}N_3O_3S$ $[M + H]^+$ 354.0907, found 354.0900. HPLC purity, 97.5% at 254 nm (t_R = 4.16 min).

2-((1-(Cyclohexanecarbonyl)-3-cyclohexyl-1*H*-1,2,4-triazol-5-yl)thio)-1-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)ethan-1-one (**17**). Yield, 42%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.23–1.35 (m, 6 H), 1.47–1.57 (m, 4 H), 1.63–1.66 (m, 1 H), 1.74–1.78 (m, 3 H), 1.86–1.89 (m, 2 H), 1.96–2.06 (m, 4 H), 2.42–2.49 (m, 1 H), 2.73–2.81 (m, 1 H), 4.29–4.32 (m, 2 H), 4.35–4.37 (m, 2 H), 5.66 (s, 2 H), 6.98 (d, J = 9.1 Hz, 1 H), 7.50–7.53 (m, 2 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.20, 25.83, 26.19, 26.33, 30.08, 33.63, 39.68, 47.66, 58.40, 64.20, 64.94, 117.94, 118.10, 122.74, 127.30, 144.01, 149.84, 167.16, 170.63, 186.38, 186.66 ppm. HRMS (ESI) m/z calculated for $C_{25}H_{32}N_3O_4S$ $[M + H]^+$ 470.2108, found 470.2101. HPLC purity, 99.5% at 254 nm (t_R = 2.52 min).

1-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-((3-(pyridin-4-yl)-1*H*-1,2,4-triazol-5-yl)thio)ethan-1-one (**18**). Yield, 47%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.30–4.32 (m, 2 H), 4.35–4.37 (m, 2 H), 4.96 (s, 2 H), 7.03 (d, J = 8.4 Hz, 1 H), 7.56–7.60 (m, 2 H), 8.31 (d, J = 5.7 Hz, 2 H), 8.9 (d, J = 6.6 Hz, 2 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 39.94 (under DMSO peak, confirmed by 2D NMR experiment ^{1}H-^{13}C HSQC), 63.95, 64.62, 117.30, 117.55, 122.32 (2 × Ar-C, pyridine, confirmed 2D NMR experiment ^{1}H-^{13}C HSQC), 122.47, 128.68, 143.20, 143.64 (2 × Ar-C, pyridine, confirmed 2D NMR experiment ^{1}H-^{13}C HSQC), 148.42, 157.00 (Ar-C, pyridine, confirmed 2D NMR experiment ^{1}H-^{13}C HMBC), 191.40 ppm. HRMS (ESI) m/z calculated for $C_{17}H_{15}N_4O_3S$ $[M + H]^+$ 355.0859, found 355.0860. HPLC purity, 99.2% at 254 nm (t_R = 2.79 min).

2-((1*H*-Imidazol-2-yl)thio)-1-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)ethan-1-one (**19**). Yield, 74%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.29–4.31 (m, 2 H), 4.34–4.36 (m, 2 H), 4.99 (s, 2 H), 7.04 (dd, J = 8.1, 0.7 Hz, 1 H), 7.51 (d, J = 2.0 Hz, 1 H), 7.53 (dd, J = 8.5, 2.1 Hz, 1 H), 7.72 (s, 2 H), 14.43 (bs, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 41.64, 64.01, 64.71, 117.44, 117.67, 121.14 (2 × C, confirmed 2D NMR experiment ^{1}H-^{13}C HSQC), 122.64, 128.08, 139.92, 143.31, 148.75, 191.12 ppm. HRMS (ESI) m/z calculated for $C_{13}H_{13}N_2O_3S$ $[M + H]^+$ 277.0647, found 277.0646. HPLC purity, 100% at 254 nm (t_R = 2.14 min).

1-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-((1-methyl-1*H*-imidazol-2-yl)thio)ethan-1-one (**20**). Yield, 93%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.83 (s, 3 H), 4.28–4.31 (m, 2 H), 4.34–4.36 (m, 2 H), 4.91 (s, 2 H), 7.02 (dd, J = 8.2, 0.5 Hz, 1 H), 7.48–7.53 (m, 2 H), 7.75 (d, J = 2.0 Hz, 1 H), 7.82 (d, J = 2.0 Hz, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 35.20, 42.32, 63.98, 64.69, 117.42, 117.65, 120.95, 122.63, 125.28, 128.02, 139.68, 143.30, 148.76, 191.44

ppm. HRMS (ESI) m/z calculated for $C_{14}H_{15}N_2O_3S$ $[M + H]^+$ 291.0803, found 291.0799. HPLC purity, 100% at 254 nm (t_R = 2.51 min).

1-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-((1-methyl-1*H*-tetrazol-5-yl)thio)ethan-1-one (**21**). Yield, 79%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.00 (s, 3 H), 4.29–4.31 (m, 2 H), 4.34–4.36 (m, 2 H), 5.01 (s, 2 H), 7.01 (d, *J* = 8.4 Hz, 1 H), 7.53 (d, *J* = 2.0 Hz, 1 H), 7.56 (dd, *J* = 8.5, 2.1 Hz, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 33.70, 41.09, 63.96, 64.65, 117.34, 117.53, 122.56, 128.52, 143.28, 148.56, 153.33, 190.91 ppm. HRMS (ESI) m/z calculated for $C_{12}H_{13}N_4O_3S$ $[M + H]^+$ 293.0708, found 293.0703. HPLC purity, 99.3% at 254 nm (t_R = 3.43 min).

1-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-(phenylthio)ethan-1-one (**22**). The product was synthesized according to the general procedure for synthesis of the final compounds, and then purified by flash chromatography using EtOAc/hex (1:3, v/v) as mobile phase. Yield, 12%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.19 (s, 2 H), 4.25–4.27 (m, 2 H), 4.29–4.31 (m, 2 H), 6.89 (dd, *J* = 8.4, 0.5 Hz, 1 H), 7.16–7.22 (m, 1 H), 7.24–7.31 (m, 2 H), 7.35–7.39 (m, 2 H), 7.47 (dd, *J* = 8.4, 2.1 Hz, 1 H), 7.49 (dd, *J* = 2.1, 0.4 Hz, 1 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 41.09, 64.20, 64.83, 117.45, 118.32, 123.00, 127.07, 129.15, 129.26, 130.42, 135.12, 143.50, 148.51, 192.68 ppm. HRMS (ESI) m/z calculated for $C_{16}H_{15}O_3S$ $[M + H]^+$ 287.0742, found 287.0747. HPLC purity, 85.2% at 254 nm (t_R = 4.88 min).

1-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-(thiophen-2-ylthio)ethan-1-one (**23**). The product was synthesized according to the general procedure for synthesis of the final compounds, and then purified by flash chromatography using EtOAc/hex (1:3, v/v) as mobile phase. Yield, 7%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.09 (s, 2 H), 4.27–4.29 (m, 2 H), 4.31–4.33 (m, 2 H), 6.89 (d, *J* = 8.4 Hz, 1 H), 6.95 (dd, *J* = 5.4, 3.6 Hz, 1 H), 7.12 (dd, *J* = 3.6, 1.3 Hz, 1 H), 7.36 (dd, *J* = 5.4, 1.3 Hz, 1 H), 7.43 (dd, *J* = 8.4, 2.2 Hz, 1 H), 7.46 (d, *J* = 2.1 Hz, 1 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 45.24, 64.21, 64.85, 117.45, 118.29, 122.99, 127.80, 129.31, 130.63, 132.49, 135.34, 143.52, 148.52, 192.53 ppm. HRMS (ESI) m/z calculated for $C_{14}H_{13}O_3S_2$ $[M + H]^+$ 293.0306, found 293.0303. HPLC purity, 95.3% at 254 nm (t_R = 4.77 min).

1-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-(thiazol-2-ylthio)ethan-1-one (**24**). Yield, 55%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.29–4.31 (m, 2 H), 4.34–4.36 (m, 2 H), 4.90 (s, 2 H), 7.00 (d, *J* = 8.4 Hz, 1 H), 7.53 (d, *J* = 2.1 Hz, 1 H), 7.56 (dd, *J* = 8.4, 2.2 Hz, 1 H), 7.63 (d, *J* = 3.4 Hz, 1 H), 7.67 (d, *J* = 3.4 Hz, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 41.03, 63.91, 64.58,

117.24, 117.49, 120.43, 122.50, 128.76, 142.58, 143.20, 148.35, 162.97, 191.32 ppm. HRMS (ESI) m/z calculated for $C_{13}H_{11}NO_3S_2$ $[M + H]^+$ 294.0259, found 294.0262. HPLC purity, 98.8% at 254 nm (t_R = 4.17 min).

Synthesis of 2-(2-((2-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-2-oxoethyl)thio)-1H-imidazol-1-yl)acetonitrile (**25**): To a suspension of **19** (0.080 g, 0.29 mmol, 1.0 equiv.) in MeCN (10 mL), K_2CO_3 (0.120 g, 0.87 mmol, 3.0 equiv.) and chloroacetonitrile (0.055 mL, 0.87 mmol, 3.0 equiv.) were added, and the reaction mixture was stirred at reflux for 18 h. The solvent was evaporated under reduced pressure, and the residue was dissolved in a mixture of DCM (50 mL) and saturated $NaHCO_3$(aq) (50 mL), and transferred into a separating funnel. The organic phase was washed with saturated brine (50 mL), dried over Na_2SO_4, filtered and evaporated under reduced pressure. The product was purified by flash chromatography using DCM/MeOH (50:1, v/v) as mobile phase. Yield, 19%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.25–4.28 (m, 2 H), 4.30–4.32 (m, 2 H), 4.45 (s, 2 H), 5.16 (s, 2 H), 6.88 (d, *J* = 8.4 Hz, 1 H), 7.12 (d, *J* = 1.4 Hz, 1 H), 7.15 (d, *J* = 1.4 Hz, 1 H), 7.40 (dd, *J* = 8.5, 2.2 Hz, 1 H), 7.44 (d, *J* = 1.9 Hz, 1 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 34.67, 43.09, 64.18, 64.85, 114.13, 117.63, 118.02, 121.44, 122.77, 128.87, 131.37, 140.70, 143.68, 148.97, 191.91 ppm. HRMS (ESI) m/z calculated for $C_{15}H_{14}O_3N_3S$ $[M + H]^+$ 316.0750, found 316.0739. HPLC purity, 99.5% at 254 nm (t_R = 2.52 min).

Synthesis of 1-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-2-((4-isopropyl-4H-1,2,4-triazol-3-yl)thio)ethan-1-ol (**26**): To a solution of **Z9** (0.225 g, 0.704 mmol, 1.0 equiv.) in a mixture of anhydrous EtOH/THF (25 mL + 25 mL), $NaBH_4$ (0.040 g, 1.057 mmol, 1.5 equiv.) was added, and the reaction mixture was stirred at room temperature for 2 h. Water (2 mL) was added and the mixture was stirred for 5 min before the solvent was evaporated off under reduced pressure. The residue was dissolved in EtOAc (50 mL) and washed with water (50 mL). The water phase was extracted with EtOAc (40 mL). The combined organic phases were washed with saturated brine (50 mL), dried over Na_2SO_4, filtered and evaporated under reduced pressure. The product was purified by flash chromatography using DCM/MeOH (20:1, v/v) as mobile phase. Yield, 87%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.49 (dd, *J* = 6.7, 3.4 Hz, 6 H), 3.43 (dd, *J* = 14.4, 8.0 Hz, 1 H), 3.53 (dd, *J* = 14.4, 3.2 Hz, 1 H), 4.25 (s, 4 H), 4.34 (hept, *J* = 6.7 Hz, 1 H), 5.06 (dd, *J* = 8.0, 3.1 Hz, 1 H), 5.15 (bs, 1 H), 6.84 (d, *J* = 8.3 Hz, 1 H), 6.91 (ddd, *J* = 8.3, 2.0, 0.6 Hz, 1 H), 6.97 (d, *J* = 2.0 Hz, 1 H), 8.19 (s, 1 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.93, 23.05, 41.89, 48.41, 64.42, 64.43, 73.31, 115.01, 117.22,

119.05, 136.48, 141.42, 143.12, 143.52, 150.78 ppm. HRMS (ESI) m/z calculated for $C_{15}H_{20}N_3O_3S$ $[M + H]^+$ 322.1225, found 322.1222. HPLC purity, 98.3% at 254 nm (t_R = 2.82 min).

Synthesis of (Z)-1-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-2-((4-isopropyl-4H-1,2,4-triazol-3-yl)thio)ethan-1-one O-methyl oxime (**27**): To a solution of **Z9** (0.110 g, 0.376 mmol, 1.0 equiv.) in absolute EtOH (10 mL), pyridine (0.45 mL, 5.64 mmol, 15.0 equiv.) and *O*-methylhydroxylamine hydrochloride (0.094 g, 1.128 mmol, 3.0 equiv.) were added. The reaction mixture was stirred at 90 °C for 16 h. The solvent was evaporated off under reduced pressure, and the residue was dissolved in DCM (50 mL) and washed with 1 M HCl (30 mL), then saturated brine (30 mL), dried over Na_2SO_4, filtered and evaporated under reduced pressure. The product was purified by flash chromatography using EtOAc as mobile phase. Yield, 66%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.36 (d, *J* = 6.8 Hz, 6 H), 3.92 (s, 3 H), 4.19–4.25 (m, 4 H), 4.28 (hept, *J* = 6.7 Hz, 1 H), 4.38 (s, 2 H), 6.79 (d, *J* = 8.5 Hz, 1 H), 7.11 (dd, *J* = 8.5, 2.2 Hz, 1 H), 7.21 (d, *J* = 2.2 Hz, 1 H), 8.17 (s, 1 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 23.10, 27.32, 48.06, 62.47, 64.25, 64.53, 115.22, 117.40, 119.68, 126.98, 141.57, 143.60, 145.08, 149.13, 152.62 ppm. HRMS (ESI) m/z calculated for $C_{16}H_{21}N_4O_3S$ $[M + H]^+$ 349.1329, found 349.1323. HPLC purity, 95.3% at 254 nm (t_R = 4.10 min).

Synthesis of 1-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-2-((4-isopropyl-4H-1,2,4-triazol-3-yl)thio)ethan-1-amine (**28**): A solution of **Z9** (0.150 g, 0.47 mmol, 1.0 equiv.) and NH_4OAc (0.404 g, 4.70 mmol, 10.0 equiv.) in MeOH (15 mL) was stirred at room temperature for 10 min, and $NaBH_3CN$ (0.030 g, 0.47 mmol, 1.0 equiv.) was added. The reaction mixture was stirred at 60 °C for 20 h. TLC analysis showed the presence of the starting compound, therefore more NH_4OAc (0.404 g, 4.70 mmol, 10.0 equiv.) and $NaBH_3CN$ (0.030 g, 0.47 mmol, 1.0 equiv.) were added, and the reaction mixture was stirred at 70 °C for 20 h. The solvent was evaporated under reduced pressure, and the residue was dissolved in a mixture of DCM (30 mL) and saturated $NaHCO_3$(aq) (30 mL), and transferred into a separating funnel. The organic phase was washed with saturated brine (50 mL), dried over Na_2SO_4, and filtered and evaporated under reduced pressure. The product was purified by flash chromatography using DCM/MeOH (9:1, v/v) as mobile phase. Yield, 50%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.45 (dd, *J* = 6.7, 2.1 Hz, 6 H), 3.32 (dd, *J* = 13.3, 8.4 Hz, 1 H), 3.55 (dd, *J* = 13.3, 5.0 Hz, 1 H), 4.22 (s, 4 H), 4.25 (dd, *J* = 8.3, 5.0 Hz, 1 H), 4.32 (hept, *J* = 6.7 Hz, 1 H), 6.80 (d, *J* = 8.3 Hz, 1 H), 6.85 (ddd, *J* = 8.3, 2.1, 0.4 Hz, 1 H), 6.90 (d, *J* = 2.1 Hz, 1 H), 8.19 (s, 1 H) ppm,

resonance for NH_2 missing. ^{13}C NMR (100 MHz, $CDCl_3$) δ 23.04, 41.98, 48.00, 54.52, 64.41 (2 × C), 115.32, 117.34, 119.55, 137.01, 141.41, 143.02, 143.57, 149.64 ppm. HRMS (ESI) m/z calculated for $C_{15}H_{21}N_4O_2S$ $[M + H]^+$ 321.1380, found 321.1373. HPLC purity, 96.02% at 254 nm (t_R = 2.23 min).

Synthesis of 2,4-dihydro-3H-1,2,4-triazol-3-one (**29**): A mixture of semicarbazide hydrochloride (10.0 g, 89.6 mmol. 1.0 eq) and trimethyl orthofromate (28.5 g, 269.0 mmol, 3.0 eq) in MeOH (100 mL) was stirred at room temperature for 2 h. The reaction mixture was concentrated under reduced pressure, toluene (100 mL) was added, and the mixture was cooled to 0 °C. The forming white precipitate was filtered off to obtain the pure product. Yield, 100%. ^1H NMR (400 MHz, DMSO-d_6) δ 7.90 (bs, 2 H), 11.45 (s, 1 H) [20].

Synthesis of tert-butyl 5-oxo-1,5-dihydro-4H-1,2,4-triazole-4-carboxylate (**30**): To a solution of **29** (2.0 g, 23.5 mmol, 1.0 equiv.) in DCM (20 mL), $(BOC)_2O$ (5.1 g, 23.5 mmol, 1.0 equiv.), DMAP (0.287 g, 2.35 mmol, 0.1 equiv.) and DIPEA (10.0 mL, 58.7 mmol, 2.5 equiv.) were added. The reaction was stirred at room temperature for 18 h. The reaction mixture was concentrated under reduced pressure and purified by flash chromatography using DCM/MeOH (20:1, v/v) as mobile phase. Yield, 14%. ^1H NMR (400 MHz, DMSO-d_6) δ 1.50 (s, 9 H), 7.98 (s, 1H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 27.97, 85.83, 136.53, 147.15, 153.68 ppm. ESI-MS for $C_7H_{11}N_3O$ $[M–H]^–$ 184.12.

Synthesis of tert-butyl 3-(2-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-2-oxoethoxy)-4H-1,2,4-triazole-4-carboxylate (**31**): To a solution of **30** (0.172 g, 0.934 mmol, 1.2 equiv.) in anhydrous DMF (3 mL) under an argon atmosphere, NaH (0.040 mg, 1.01 mmol, 1.3 equiv.) was added and stirred at room temperature for 15 min, followed by addition of bromide **6** (0.200 g, 0.778 mmol, 1.0 equiv.). The reaction mixture was stirred at room temperature for 18 h. EtOAc (60 mL) was then added, and the organic phase was washed with water (2 × 30 mL) and brine (30 mL), and dried over Na_2SO_4, and the solvent was evaporated under reduced pressure. The product was purified by flash chromatography using DCM/MeOH (50:1, v/v) as mobile phase. Yield, 47%. ^1H NMR (400 MHz, $CDCl_3$) δ 1.47 (s, 9 H), 4.29–4.36 (m, 4 H), 5.04 (s, 2 H), 6.96 (d, J = 9.1 Hz, 1 H), 7.50–7.52 (m, 2 H), 7.62 (s, 1 H) ppm. ESI-MS for $C_{17}H_{19}N_3O_6$ $[M + Na]^+$ 384.31; $[M – H]^–$ 360.37.

Synthesis of 2-((4H-1,2,4-triazol-3-yl)oxy)-1-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)ethan-1-one (**32**): To a solution of **31** (0.100 g, 0.276 mmol, 1.0 equiv.) in DCM (5 mL), TFA (1 mL)

was added drop-wise. The mixture was stirred at room temperature for 2 h, then the solvent was evaporated off under reduced pressure. Further, 1 mL of 2 M HCl solution in ether was added, forming of precipitate, followed by addition of saturated NaOH solution in MeOH. The solvent was evaporated under reduced pressure, and the product was purified by flash chromatography using DCM/MeOH (20:1, v/v) as mobile phase. Additionally, the product was recrystallized from DCM. Yield, 58%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.29–4.31 (m, 2 H), 4.34–4.36 (m, 2 H), 5.12 (s, 2 H), 7.03 (d, J = 8.4 Hz, 1 H), 7.54 (d, J = 2.1 Hz, 1 H), 7.58 (dd, J = 8.4, 2.1 Hz, 1 H), 7.79 (d, J = 1.4 Hz, 1 H), 11.70 (s, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 47.39, 63.95, 64.64, 117.03, 117.45, 122.08, 127.67, 138.63, 143.39, 148.62, 154.59, 191.19 ppm. ESI-MS for $C_{12}H_{11}N_3O_4$ $[M + H]^+$ 262.3, $[M – H]^–$ 260.23. HRMS (ESI) m/z calculated for $C_{12}H_{12}N_3O_4$ $[M + H]^+$ 262.0828, found 262.0821. HPLC purity, 98.8% at 254 nm (t_R = 2.06 min).

Synthesis of 2-(5-amino-1H-1,2,4-triazol-1-yl)-1-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)ethan-1-one (**33**): To a suspension of bromide **6** (0.200 g, 0.778 mmol, 1.0 equiv.) and 4*H*-1,2,4-triazol-3-amine (0.068 g, 0.778 mmol, 1.0 equiv.) in anhydrous EtOH (15 mL), K_2CO_3 (0.161 g, 1.167 mml, 1.5 equiv.) was added, and the reaction was stirred at room temperature for 18 h. The solvent was evaporated under reduced pressure, the residue was dissolved in EtOAc (50 mL), and the organic phase was washed with water (3 × 25 mL). The organic phase was evaporated under reduced pressure and the product was purified by flash chromatography using DCM/MeOH (9:1, v/v) as mobile phase. Yield, 20%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.30–4.32 (m, 2 H), 4.34–4.36 (m, 2 H), 5.45 (s, 2 H), 6.16 (s, 2 H), 7.03 (d, J = 8.4 Hz, 1 H), 7.34 (s, 1H), 7.52–7.55 (m, 2 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 52.50, 63.93, 64.59, 117.10, 117.29, 122.01, 128.06, 143.25, 148.32, 148.49, 156.26, 190.92 ppm. HRMS (ESI) m/z calculated for $C_{12}H_{12}N_4O_3$ $[M + H]^+$ 261.0988, found 261.0985. HPLC purity, 97.1% at 254 nm (t_R = 2.04 min).

General procedure for the synthesis of compounds **34a-c** [15]: 3-Aminotriazole (3.36 g, 40.0 mmol, 1.0 equiv.) was suspended in DMFDMA reagent (20 mL), and the mixture was stirred at 110 °C for 18 h. The solvent was evaporated under reduced pressure, the isomers **34a-c** were separated and purified by flash chromatography using DCM/MeOH = 9/1 (V/V) as a mobile phase.

(*E*)-*N*,*N*-Dimethyl-*N'*-(1-methyl-1*H*-1,2,4-triazol-3-yl)formimidamide (**34a**). Yield (isolated), 15%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.90 (s, 3 H), 3.01 (s, 3 H), 3.59 (s, 3 H), 7.51 (s, 1 H), 8.37 (s, 1 H) ppm. ESI-MS for $C_6H_{11}N_5$ $[M+H]^+$ 153.9; $[M + Na]^+$ 175.9.

(*E*)-*N*,*N*-Dimethyl-*N'*-(1-methyl-1*H*-1,2,4-triazol-5-yl)formimidamide (**34b**). Yield (isolated), 50%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.99 (d, J = 0.4 Hz, 3 H), 3.09 (s, 3 H), 3.68 (s, 3 H), 8.07 (s, 1 H), 8.26 (s, 1 H) ppm. ESI-MS for $C_6H_{11}N_5$ $[M + H]^+$ 153.9.

(*E*)-*N*,*N*-Dimethyl-*N'*-(4-methyl-4*H*-1,2,4-triazol-3-yl)formimidamide (**34c**). Yield (isolated), 2%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.97 (s, 3 H), 3.08 (s, 3 H), 3.39 (s, 3 H), 8.10 (s, 1 H), 8.33 (s, 1 H) ppm. ESI-MS for $C_6H_{11}N_5$ $[M+H]^+$ 154.1. **34c** was not used in the next reaction steps due to low amounts of the product yielded after purification.

General procedure for the synthesis of compounds **35a-b** [15]: Compound **34a** or **34b** (1.0 equiv.) was suspended in 4 M NaOH(aq) (50 mL) and heated under reflux for 1 h. After cooling, the solution was neutralized with 37% HCl(aq) and extracted with EtOAc (3 × 50 mL). The combined organic phases were washed with saturated brine (50 mL), dried over Na_2SO_4, and filtered and evaporated under reduced pressure, to obtain pure products.

1-Methyl-1*H*-1,2,4-triazol-3-amine (**35a**). Yield, 66%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.59 (d, J = 0.5 Hz, 3 H), 5.19 (bs, 2 H), 7.87 (s, 1 H) ppm. ESI-MS for $C_3H_6N_4$ $[M+MeOH+H]^+$ 130.9.

1-Methyl-1*H*-1,2,4-triazol-5-amine (**35b**). Yield, 36%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.48 (s, 3 H), 6.11 (bs, 2 H), 7.27 (s, 1 H) ppm. ESI-MS for $C_3H_6N_4$ $[M+MeOH+H]^+$ 130.9.

3-Amino-4-(2-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-oxoethyl)-1-methyl-1*H*-1,2,4-triazol-4-ium bromide (**36a**). Synthesized following the general procedure for the synthesis of thiols. The product was purified by flash chromatography using DCM/MeOH (9:1, v/v) as mobile phase. Yield, 41%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.90 (d, J = 0.9 Hz, 3 H), 4.31–4.33 (m, 2 H), 4.36–4.38 (m, 2 H), 5.71 (s, 2 H), 7.09 (d, J = 8.4 Hz, 1 H), 7.31 (bs, 2 H), 7.54 (d, J = 2.0 Hz, 1 H), 7.56 (dd, J = 8.4, 2.1 Hz, 1 H), 9.32 (d, J = 1.0 Hz, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 38.26, 51.01, 64.03, 64.75, 117.33, 117.53, 122.40, 127.12, 139.82, 143.38, 148.91, 154.96, 188.90 ppm. HRMS (ESI) m/z calculated for $C_{13}H_{15}O_3N_4$ $[M + H]^+$ 275.1139, found 275.1127. HPLC purity, 96.7% at 254 nm (t_R = 1.79 min).

5-Amino-4-(2-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-oxoethyl)-1-methyl-1*H*-1,2,4-triazol-4-ium bromide (**36b**). Synthesized following the general procedure for the synthesis of thiols. The product was purified by flash chromatography using DCM/MeOH (9:1, v/v) as mobile phase. Yield, 33%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.71 (s, 3 H), 4.31–4.33 (m, 2 H), 4.36–4.38 (m, 2 H), 5.64 (s, 2 H), 7.09 (d, *J* = 8.4 Hz, 1 H), 7.53 (d, *J* = 2.0 Hz, 1 H), 7.56 (dd, *J* = 8.4, 2.2 Hz, 1 H), 8.36 (s, 1 H), 8.54 (bs, 2 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 35.14, 50.99, 64.01, 64.73, 117.25, 117.52, 122.34, 127.11, 140.26, 143.38, 148.87, 149.53, 188.98 ppm. HRMS (ESI) m/z calculated for $C_{13}H_{15}O_3N_4$ $[M + H]^+$ 275.1139, found 275.1128. HPLC purity, 98.0% at 254 nm (t_R = 1.96 min).

1-(1*H*-Indol-3-yl)-2-((4-isopropyl-4*H*-1,2,4-triazol-3-yl)thio)ethanone (**37**). Synthesized following the general procedure for the synthesis of the final products. Yield, 10%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.46 (d, *J* = 6.7 Hz, 6 H), 4.54 (hept, *J* = 6.7 Hz, 1 H), 4.81 (s, 2 H), 7.22 (pd, *J* = 7.1, 1.4 Hz, 2 H), 7.49–7.51 (m, 1 H), 8.11 (dd, *J* = 7.0, 1.7 Hz, 1 H), 8.48 (d, *J* = 3.2 Hz, 1 H), 9.37 (s, 1 H), 12.16 (s, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 22.10, 40.97, 49.44, 112.39, 114.49, 121.11, 122.17, 123.18, 125.30, 135.17, 136.59, 142.84 (confirmed by 2D NMR experiment ^{1}H-^{13}C HSQC), 150.08 (confirmed by 2D NMR experiment ^{1}H-^{13}C HMBC), 187.00 ppm. HRMS (ESI) m/z calculated for $C_{15}H_{17}ON_4S$ $[M + H]^+$ 301.1118, found 301.1121. HPLC purity, 96.4% at 254 nm (t_R = 3.46 min).

2-((4-Isopropyl-4*H*-1,2,4-triazol-3-yl)thio)-1-(2-methyl-1*H*-indol-3-yl)ethanone (**38**). Synthesized following the general procedure for the synthesis of the final products. Yield, 62%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.52 (d, *J* = 6.7 Hz, 6 H), 2.75 (s, 3 H), 4.61 (hept, *J* = 6.7 Hz, 1 H), 5.11 (s, 2 H), 7.15–7.20 (m, 2 H), 7.40–7.44 (m, 1 H), 7.98–8.03 (m, 1 H), 9.68 (s, 1 H), 12.38 (s, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 15.26, 21.90, 45.68, 50.15, 111.33, 111.59, 120.52, 121.86, 122.26, 126.55, 134.86, 142.68, 146.04, 150.93, 186.75 ppm. HRMS (ESI) m/z calculated for $C_{16}H_{19}ON_4S$ $[M + H]^+$ 315.1274, found 315.1276. HPLC purity, 98.7% at 254 nm (t_R = 3.61 min).

1-(Benzo[*d*][1,3]dioxol-5-yl)-2-((4-isopropyl-4*H*-1,2,4-triazol-3-yl)thio)ethanone (**39**). Synthesized following the general procedure for the synthesis of the final products. Yield, 66%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.50 (d, *J* = 6.7 Hz, 6 H), 4.57 (hept, *J* = 6.7 Hz, 1 H), 5.02 (s, 2 H), 6.17 (s, 2 H), 7.09 (d, *J* = 8.2 Hz, 1 H), 7.50 (d, *J* = 1.8 Hz, 1 H), 7,69 (dd, *J* = 8.2, 1.8 Hz, 1 H), 9.68 (s, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 21.81, 41.26, 50.12, 102.25, 107.66, 108.24, 125.26, 129.34, 142.88, 147.88, 150.41, 152.11, 190.44 ppm. HRMS

(ESI) m/z calculated for $C_{14}H_{16}O_3N_3S$ $[M + H]^+$ 306.0907, found 306.0909. HPLC purity, 97.9% at 254 nm (t_R = 3.58 min).

2-((4-Isopropyl-4*H*-1,2,4-triazol-3-yl)thio)-1-(4-methoxyphenyl)ethanone (**40**). Synthesized following the general procedure for the synthesis of the final products. Yield, 60%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.49 (d, *J* = 6.7 Hz, 6 H), 3.86 (s, 3 H), 4.55 (hept, *J* = 6.7 Hz, 1 H), 5.03 (s, 2 H), 7.09 (d, *J* = 8.9 Hz, 2 H), 8.00 (d, *J* = 8.9 Hz, 2 H), 9.57 (s, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 21.87, 41.44, 50.37, 55.77, 114.20, 127.75, 130.98, 142.97, 150.78, 163.84, 190.78 ppm. HRMS (ESI) m/z calculated for $C_{14}H_{18}O_2N_3S$ $[M + H]^+$ 292.1114, found 292.1116. HPLC purity, 99.6% at 254 nm (t_R = 3.71 min).

2-((4-Isopropyl-4*H*-1,2,4-triazol-3-yl)thio)-1-(3-methoxyphenyl)ethanone (**41**). Synthesized following the general procedure for the synthesis of the final products. Yield, 60%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.50 (d, *J* = 6.7 Hz, 6 H), 3.83 (s, 3 H), 4.57 (hept, *J* = 6.7 Hz, 1 H), 5.10 (s, 2 H), 7.28 (ddd, *J* = 8.2, 2.6, 1.0 Hz, 1 H), 7.49 (t, *J* = 7.9 Hz, 1 H), 7.50 (dd, *J* = 2.6, 1.5 Hz, 1 H), 7.63 (dt, *J* = 7.8, 1.2 Hz, 1 H), 9.64 (s, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 21.91, 41.53, 50.15, 55.52, 113.01, 120.07, 120.99, 130.17, 136.30, 142.98, 150.37, 159.48, 192.40 ppm. HRMS (ESI) m/z calculated for $C_{14}H_{18}O_2N_3S$ $[M + H]^+$ 292.1114, found 292.1117. HPLC purity, 97.7% at 254 nm (t_R = 3.77 min).

2-((4-Isopropyl-4*H*-1,2,4-triazol-3-yl)thio)-1-(2-methoxyphenyl)ethanone (**42**). Synthesized following the general procedure for the synthesis of the final products. Yield, 70%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.47 (d, *J* = 6.8 Hz, 6 H), 3.93 (s, 3 H), 4.46 (hept, *J* = 6.7 Hz, 1 H), 4.89 (s, 2 H), 6.96–7.02 (m, 2 H), 7.50 (ddd, *J* = 8.4, 7.3, 1.8 Hz, 1 H), 7.82 (dd, *J* = 7.8, 1.9 Hz, 1 H), 8.18 (s, 1 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 23.11, 46.64, 48.16, 55.84, 111.69, 120.92, 125.49, 131.19, 134.95, 141.37, 149.45, 159.30, 194.11 ppm. HRMS (ESI) m/z calculated for $C_{14}H_{18}O_2N_3S$ $[M + H]^+$ 292.1114, found 292.1116. HPLC purity, 99.5% at 254 nm (t_R = 3.79 min).

1-(4-Bromophenyl)-2-((4-isopropyl-4*H*-1,2,4-triazol-3-yl)thio)ethanone (**43**). Synthesized following the general procedure for the synthesis of the final products. Yield, 89%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.49 (d, *J* = 6.7 Hz, 6 H), 4.56 (hept, *J* = 6.7 Hz, 1 H), 5.08 (s, 2 H), 7.79 (d, *J* = 8.6 Hz, 2 H), 7.96 (d, *J* = 8.6 Hz, 2 H), 9.63 (s, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 21.93, 41.31, 50.05, 128.22, 130.49, 132.02, 134.00, 143.00, 150.13, 191.96

ppm. HRMS (ESI) m/z calculated for $C_{13}H_{15}ON_3SBr$ $[M + H]^+$ 340.0114, found 340.0118. HPLC purity, 99.3% at 254 nm (t_R = 4.25 min).

2-((4-Isopropyl-4*H*-1,2,4-triazol-3-yl)thio)-1-(*p*-tolyl)ethanone (**44**). Synthesized following the general procedure for the synthesis of the final products. Yield, 68%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.50 (d, *J* = 6.7 Hz, 6 H), 2.39 (s, 3 H) 4.57 (hept, *J* = 6.6 Hz, 1 H), 5.09 (s, 2 H), 7.38 (d, *J* = 8.0 Hz, 2 H), 7.93 (d, *J* = 8.3 Hz, 2 H), 9.73 (s, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 21.30, 21.88, 41.52, 50.25, 128.63, 129.49, 132.44, 142.98, 144.70, 150.57, 192.01 ppm. HRMS (ESI) m/z calculated for $C_{14}H_{18}ON_3S$ $[M + H]^+$ 276.1165, found 276.1168. HPLC purity, 98.0% at 254 nm (t_R = 3.99 min).

2-((4-Isopropyl-4*H*-1,2,4-triazol-3-yl)thio)-1-phenylethanone (**45**). Synthesized following the general procedure for the synthesis of the final products. Yield, 48%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.49 (d, *J* = 6.7 Hz, 6 H), 4.56 (hept, *J* = 6.7 Hz, 1 H), 5.10 (s, 2 H), 7.55–7.60 (m, 2 H), 7.69–7.73 (m, 1 H), 8.02–8.05 (m, 2 H), 9.59 (s, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 21.94, 41.39, 49.92, 128.48, 128.93, 134.04, 134.96, 142.95, 150.11, 192.59 ppm. HRMS (ESI) m/z calculated for $C_{13}H_{16}ON_3$ $[M + H]^+$ 262.1009, found 262.1009. HPLC purity, 97.1% at 254 nm (t_R = 3.56 min).

1-(4-(Dimethylamino)phenyl)-2-((4-isopropyl-4*H*-1,2,4-triazol-3-yl)thio)ethanone (**46**). Synthesized following the general procedure for the synthesis of the final products. Yield, 52%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.49 (d, *J* = 6.7 Hz, 6 H), 3.04 (s, 6 H), 4.56 (hept, *J* = 6.7 Hz, 1 H), 4.97 (s, 2 H), 6.75 (d, *J* = 9.2 Hz, 2 H), 7.84 (d, *J* = 9.1 Hz, 2 H), 9.63 (s, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 21.90, 39.66, 41.29, 50.08, 110.78, 122.01, 130.62, 142.87, 150.69, 153.76, 189.42 ppm. HRMS (ESI) m/z calculated for $C_{15}H_{21}ON_4S$ $[M + H]^+$ 305.1431, found 305.1432. HPLC purity, 95.2% at 254 nm (t_R = 3.88 min).

4-(2-((4-Isopropyl-4*H*-1,2,4-triazol-3-yl)thio)acetyl)benzonitrile (**47**). Synthesized following the general procedure for the synthesis of the final products. Yield, 90%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.49 (d, *J* = 6.7 Hz, 6 H), 4.56 (hept, *J* = 6.6 Hz, 1 H), 5.13 (s, 2 H), 8.07 (d, *J* = 8.6 Hz, 2 H), 8.18 (d, *J* = 8.7 Hz, 2 H), 9.66 (s, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 21.89, 41.54, 50.29, 115.78, 118.08, 129.13, 132.96, 138.19, 143.08, 150.26, 192.15 ppm. HRMS (ESI) m/z calculated for $C_{14}H_{15}ON_4S$ $[M + H]^+$ 287.0961, found 287.0964. HPLC purity, 93.6% at 254 nm (t_R = 3.41 min).

1-((4-Isopropyl-4*H*-1,2,4-triazol-3-yl)thio)-3,3-dimethylbutan-2-one (**48**). Synthesized following the general procedure for the synthesis of the final products. The product was purified by flash chromatography using EtOAc/MeOH (95:5, v/v) as mobile phase. Yield, 5%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.22 (s, 9 H), 1.49 (d, *J* = 6.7 Hz, 6 H), 4.42 (hept, *J* = 6.7 Hz, 1 H), 4.55 (s, 2 H), 8.19 (s, 1 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 23.05, 26.68, 40.90, 44.50, 48.24, 141.47, 149.34, 209.15 ppm. HRMS (ESI) m/z calculated for $C_{11}H_{20}ON_3S$ $[M + H]^+$ 242.1322, found 242.1323. HPLC purity, 96.0% at 254 nm (t_R = 3.35 min).

2-((4-Isopropyl-4*H*-1,2,4-triazol-3-yl)thio)-1-(3-nitrophenyl)ethanone (**49**). Synthesized following the general procedure for the synthesis of the final products. Yield, 86%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.50 (d, *J* = 6.7 Hz, 6 H), 4.58 (hept, *J* = 6.6 Hz, 1 H), 5.19 (s, 2 H), 7.89 (t, *J* = 8.0 Hz, 1 H), 8.47 (dt, *J* = 7.8, 1.4 Hz, 1 H), 8.53 (ddd, *J* = 8.2, 2.4, 1.0 Hz, 1 H), 8.71 (t, *J* = 2.0 Hz, 1 H), 9.65 (s, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 21.96, 41.42, 50.08, 122.85, 128.13, 130.84, 134.70, 136.23, 143.08, 148.05, 149.94, 191.53 ppm. HRMS (ESI) m/z calculated for $C_{13}H_{15}O_3N_4S$ $[M + H]^+$ 307.0859, found 307.0861. HPLC purity, 98.6% at 254 nm (t_R = 3.63 min).

1-(4-Hydroxyphenyl)-2-((4-isopropyl-4*H*-1,2,4-triazol-3-yl)thio)ethanone (**50**). Synthesized following the general procedure for the synthesis of the final products. Yield, 84%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.50 (d, *J* = 6.7 Hz, 6 H), 4.58 (hept, *J* = 6.6 Hz, 1 H), 5.05 (s, 2 H), 6.90 (d, *J* = 8.8 Hz, 2 H), 7.90 (d, *J* = 8.8 Hz, 2 H), 9.82 (s, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 21.85, 41.51, 50.48, 115.53, 126.35, 131.23, 142.97, 150.98, 162.93, 190.40 ppm. HRMS (ESI) m/z calculated for $C_{13}H_{16}O_2N_3S$ $[M + H]^+$ 278.0958, found 278.0960. HPLC purity, 98.9% at 254 nm (t_R = 2.76 min).

*General procedure for alkylation of phenol **50***: To a solution of **50** (0.15 g, 0.54 mmol, 1.0 equiv.) in MeCN (10 mL), the corresponding alkyl halide (1.5 equiv.) and K_2CO_3 (2.0 equiv.) were added. The resulting suspension was stirred at 50 °C overnight. The solvent was evaporated under reduced pressure, and the residue was dissolved in a mixture of DCM (30 mL) and saturated $NaHCO_3$(aq) (30 mL), and transferred into a separating funnel. The organic phase was washed with saturated brine (50 mL), dried over Na_2SO_4, filtered and evaporated under reduced pressure. The product was purified by flash chromatography using DCM/MeOH (20:1, v/v) as mobile phase.

2-(4-(2-((4-Isopropyl-4*H*-1,2,4-triazol-3-yl)thio)acetyl)phenoxy)acetonitrile (**51**). Synthesized following the general procedure for alkylation of phenol **50**. Yield, 65%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.46 (d, *J* = 6.7 Hz, 6 H), 4.40 (hept, *J* = 6.7 Hz, 1 H), 4.86 (s, 2 H), 4.87 (s, 2 H), 7.02 (d, *J* = 9.0 Hz, 2 H), 8.02 (d, *J* = 9.0 Hz, 2 H), 8.19 (s, 1 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 23.00, 41.51, 48.30, 53.36, 114.51, 114.82, 130.19, 131.16, 141.65, 148.98, 160.60, 191.64 ppm. HRMS (ESI) m/z calculated for $C_{15}H_{17}O_2N_4S$ $[M + H]^+$ 317.1067, found 317.1064. HPLC purity, 98.0% at 254 nm (t_R = 3.45 min).

1-(4-Isopropoxyphenyl)-2-((4-isopropyl-4*H*-1,2,4-triazol-3-yl)thio)ethanone (**52**). Synthesized following the general procedure for alkylation of phenol **50**. Yield, 32%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.33 (d, *J* = 6.1 Hz, 6 H), 1.46 (d, *J* = 6.7 Hz, 6 H), 4,41 (hept, *J* = 6.7 Hz, 1 H), 4.62 (hept, *J* = 6.1 Hz, 1 H), 4.87 (s, 2 H), 6.88 (d, *J* = 9.0 Hz, 2 H), 7.94 (d, *J* = 9.0 Hz, 2 H), 8.18 (s, 1 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 21.91, 22.99, 41.76, 48.22, 70.30, 115.33, 127.64, 131.01, 141.51, 149.26, 162.80, 191.61 ppm. HRMS (ESI) m/z calculated for $C_{16}H_{22}O_2N_3S$ $[M + H]^+$ 320.1427, found 320.1429. HPLC purity, 91.9% at 254 nm (t_R = 4.41 min).

Ethyl 2-(4-(2-((4-isopropyl-4*H*-1,2,4-triazol-3-yl)thio)acetyl)phenoxy)acetate (**53**). Synthesized following the general procedure for alkylation of phenol **50**. Yield, 32%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.27 (t, *J* = 7.1 Hz, 3 H), 1.46 (d, *J* = 6.7 Hz, 6 H), 4.25 (q, *J* = 7.1 Hz, 2 H), 4.40 (hept, *J* = 6.7 Hz, 1 H), 4.66 (s, 2 H), 4.86 (s, 2 H), 6.92 (d, *J* = 9.0 Hz, 2 H), 7.97 (d, *J* = 9.0 Hz, 2 H), 8.17 (s, 1 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.20, 22.98, 41.65, 48.23, 61.71, 65.20, 114.64, 129.02, 130.98, 141.56, 149.09, 162.26, 168.09, 191.65 ppm. HRMS (ESI) m/z calculated for $C_{17}H_{22}O_4N_3S$ $[M + H]^+$ 364.1326, found 364.1324. HPLC purity, 90.4% at 254 nm (t_R = 3.86 min).

2-(4-(2-((4-Isopropyl-4*H*-1,2,4-triazol-3-yl)thio)acetyl)phenoxy)acetic acid (**54**). To a suspension of **53** (31.5 mg, 0.087 mmol, 1.0 equiv.) in MeOH (2 mL), 0.1 M NaOH(aq) (1.3 mL, 1.5 equiv.) was added and the reaction mixture was stirred at room temperature for 4 h. The mixture was acidified with 1 M HCl(aq) to pH ~1. The precipitate that formed was filtered off, yielding the pure product. Yield, 66%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.40 (d, *J* = 6.7 Hz, 6 H), 4.39 (hept, *J* = 6.9 Hz, 1 H), 4.82 (s, 2 H), 4.83 (s, 2 H), 7.04 (d, *J* = 9.0 Hz, 2 H), 7.97 (d, *J* = 9.0 Hz, 2 H), 8.72 (s, 1 H), 13.15 (s, 1 H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ 22.54, 40.67, 47.75, 64.57, 114.56, 128.49, 130.77, 142.84, 147.40, 162.04,

169.73, 191.69 ppm. HRMS (ESI) m/z calculated for $C_{15}H_{18}O_4N_3S$ $[M + H]^+$ 336.1013, found 336.1013. HPLC purity, 99.3% at 254 nm (t_R = 2.80 min).

Synthesis of 1-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-hydroxyethan-1-one (**55**, Supplementary information, Table S1). A solution of bromide **6** (0.40 g, 1.56 mmol, 1.0 equiv.) and lithium formate (0.54 g, 7.78 mmol, 5.0 equiv.) in 90% EtOH(aq) (40 mL) was heated to reflux for 12 h. After the reaction was complete, the mixture was cooled to room temperature, and ethanol was removed under reduced pressure. The residue was diluted in water (30 mL) and extracted with EtOAc (3 × 20 mL). The combined organic phases were washed with saturated brine (40 mL), dried over Na_2SO_4, filtered and evaporated under reduced pressure. The product was purified by flash chromatography using EtOAc/Hex (1:1, v/v) as mobile phase. Yield, 89%. ^{1}H NMR (400 MHz, acetone-d_6) δ 3.79 (t, *J* = 5.1 Hz, 1 H), 4.31–4.33 (m, 2 H), 4.35–4.38 (m, 2 H), 4.80 (d, *J* = 5.1 Hz, 2 H), 6.95 (d, *J* = 8.4 Hz, 1 H), 7.45 (d, *J* = 2.1 Hz, 1 H), 7.50 (dd, *J* = 8.5, 2.1 Hz, 1 H) ppm. ^{13}C NMR (100 MHz, acetone-d_6) δ 65.01, 65.64, 65.88, 117.59, 118.18, 122.42, 128.56, 144.62, 149.63, 197.87 ppm. HRMS (ESI) m/z calculated for $C_{10}H_{11}O_4$ $[M + H]^+$ 195.0657, found 195.0653. HPLC purity, 99.7% at 254 nm (t_R = 2.17 min). [21]

S-(2-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-2-oxoethyl) ethanethioate (**56**, Supplementary information, Table S1). To a cooled (0 °C) suspension of bromide **6** (1.5 g, 5.83 mmol, 1 equiv.) in DMF (15 mL), $KSCOCH_3$ was added and the reaction was stirred at 0 °C for 1 h. Then 50 mL of water was added, and the aqueous phase was extracted with DCM (2 × 50 mL). The combined organic phases were washed with water (50 mL) and brine (50 mL), and dried over Na_2SO_4. The solvent was evaporated under reduced pressure, and the product was purified by flash chromatography using DCM as mobile phase. Yield, 69%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.37 (s, 3 H), 4.28–4.30 (m, 2 H), 4.33–4.35 (m, 2 H), 4.43 (s, 2 H), 6.99 (d, *J* = 8.4 Hz, 1 H), 7.49 (d, *J* = 2.1 Hz, 1 H), 7.53 (dd, *J* = 8.5, 2.2 Hz, 1 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 30.37, 36.49, 64.19, 64.84, 117.52, 118.15, 122.83, 129.37, 143.56, 148.72, 191.74, 194.41 ppm. ESI-MS for $C_{12}H_{12}O_4S$ $[M+Na]^+$ 275.32. HRMS (ESI) m/z calculated for $C_{12}H_{13}O_4S$ $[M + H]^+$ 253.0529, found 253.0526.

Enzymes and assay buffers

Recombinant CatX was expressed in *Pichia pastoris* [22]. The assay buffer for determination of CatX activity was 100 mM sodium acetate, pH 5.5 with 5 mM cysteine, 1.5 mM EDTA,

and 0.1% PEG 8000. 0.01% Triton X-100 was added to prevent false positive inhibition due to aggregation of compounds. The enzyme was activated at 37 °C for 5 min before the assay. Recombinant cathepsins B, L and S were used to determine the possible inhibition by the selected compounds. Recombinant cathepsin S was expressed in *P. pastoris* and recombinant cathepsins B and L were expressed in *E. coli* [23, 24]. Assay buffers for determining activities of cathepsin L consisted of 100 mM sodium acetate buffer, pH 5.5, for cathepsins S and B (endopeptidase activity) of 100 mM phosphate buffer pH 6.5 and 6.0, respectively, and for cathepsin B (exopeptidase activity) of 60 mM acetate buffer, pH 5.0. Each assay buffer contained 5 mM cysteine, 1.5 mM EDTA and 0.1% PEG 8000. The fluorogenic substrates *Z*-FR-AMC, *Z*-VVR-AMC, Abz-GIVRAK(Dnp)-OH[25] and *Z*-RR-AMC were used for determining cathepsins L, S and B exo- and endo- peptidase activities, respectively. Enzymes were activated at 37 °C for 5 min before the assays.

Determination of relative inhibition and IC_{50} values

Abz-FEK(Dnp)-OH was used as a CatX specific substrate in activity assays [16]. Here, 90 μL activated CatX (20 nM) was added to the wells of a black 96-well plate with 5 μL substrate (3.25 μM final concentration) and 5 μL of the compounds (50 μM final concentration). The reaction was monitored continuously (30-45 min) at 420 nm ±10 nm, with excitation at 320 nm ±20 nm, and at 37 °C. Relative inhibition (%) was calculated according to the equation $(1 - v_i / v_0) \times 100$, where v_i is the reaction velocity in the presence of the compound, and v_0 is the reaction velocity without the compound.

For determination of the IC_{50} values, substrate cleavage was monitored at seven compound concentrations (250, 100, 30, 10, 3, 1, 0.3 μM). The compound dilutions were prepared in DMSO in such a way that there was always the same amount of DMSO (5% DMSO) in the final mixture. The assays were than performed as described above. The IC_{50} curves were analyzed using GraphPad Prism software (GraphPad Software Inc., La Jolla, San Jose, CA, USA).

Determination of the reversibility of binding

The reversibility of the binding was determined in the washout experiment, as described previously [12]. Briefly, 20 nM CatX was mixed with the inhibitor at the saturating level of 100 μM and incubated for 1 hour at room temperature. One part of the mixture was assayed for CatX activity and the other part was diluted approximately 150000-fold with assay buffer

using step-wise dilution and concentration with Centricons (10 kDa cut-off; Millipore) and then assayed for CatX activity.

Cell cultures and cytotoxicity assay

The PC-3 human prostate cancer cell line (CRL-1435; ATCC, Manassas, USA) was cultured in Advanced DMEM (Gibco) and F-12 (Gibco) (1:1, v/v) with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin. Here, 6.5 $\times 10^4$ cells/mL were seeded into the wells of a 96-well plate. The cells were treated with the compounds (10, 20 μM) for 24 h or 48 h. The cytotoxicity was determined with the MTS reagent (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega) and A_{492} was measured after 1 h, according to the manufacturer instructions.

Real-time cell-migration assay

Cell-migration assays were performed using a Real-Time Cell Analyzer Dual Plate Instrument (xCELLigence System; ACEA Biosciences), as previously described [12]. The xCELLigence CIM plates were coated with fibronectin (10 μg/mL; BD Biosciences) on the lower and upper sides of the microporous PET membrane, for 30 min at room temperature and 2 h at 37 °C, respectively. Excess fibronectin was removed and the wells were washed with phosphate-buffered saline. The compounds (10, 20 μM) were added to the complete medium (lower chambers) or to the serum-free medium (upper chambers). Here, 0.1% DMSO was used as a control, with 2×10^4 cells plated per well. The impedance data are reported as the cell index CI), and were measured continuously every 15 min for 72 h, and then analyzed with the Real-Time Cell Analyzer software (ACEA Biosciences, Inc.). The data were normalized to cells treated with DMSO.

Molecular modeling

3D structure of cathepsin X without any co-crystallized ligand (PDB: 1EF7) was used. PDB file was imported into the Maestro program (Schrödinger Release 2019-4, Schrödinger, LLC, New York, NY, 2019), where the protein structure was prepared using Protein Preparation Wizard. Chain B was removed and hydrogens were added to the structure to assume a pH 7.0 environment. Following the refinement of hydrogen bonding network, waters with fewer than 3 H-bonds to the protein were removed. The position of heavy atoms was locally minimized using OPLS3e force field. SiteMap with default settings was used to identify the active site

and one allosteric site [26]. Next, Detect shallow binding sites option in the SiteMap was used to identify additional 3 allosteric sites. A receptor grid was generated for each identified binding site. Ligands with measured residual activity were prepared using LigPrep to account for all possible tautomers at pH 7.0 ± 2.0. Docking poses were obtained through a docking protocol using Glide, with the following parameters: XP (extra precision), flexible ligand sampling, perform postdocking minimization [27]. Ligand-protein interactions were visualized and the best scoring poses were manually inspected. The docking experiments suggest binding into the active site, where best score and superposition of ligands was achieved.

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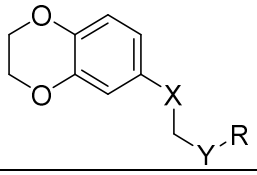
Figure 1: Schematic representation of structural alterations to **Z9**.

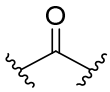
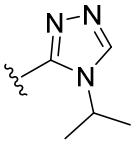
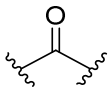
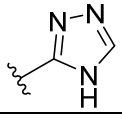
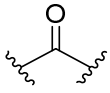
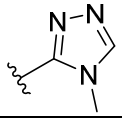
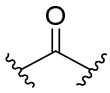
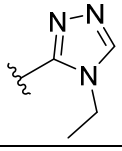
Figure 2: Crucial observations regarding structure activity relationships of CatX inhibitors.

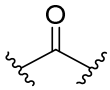
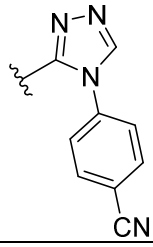
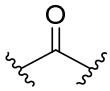
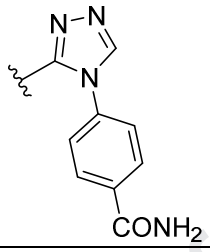
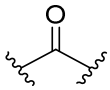
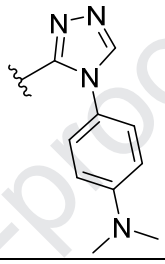
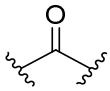
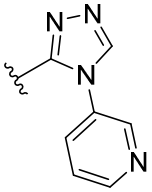
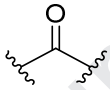
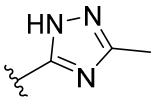
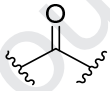
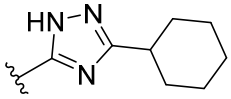
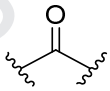
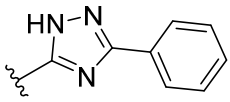
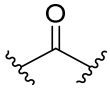
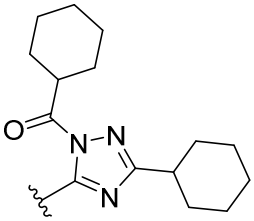
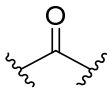
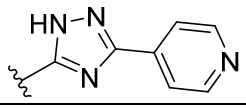
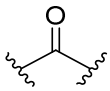
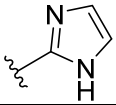
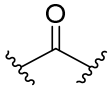
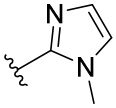
Figure 3: Cytotoxicity of the selected compounds on PC-3 cells.
MTS assay was used to analyze the cytotoxic effects of the selected compounds at 10 µM and 20 µM (as indicated), with treatments for 24 h (left) and 48 h (right). Data are means ± standard deviation from three independent assays, each performed in triplicate.

Figure 4: Compound **25** inhibits PC-3 cell migration.
Cell migration was evaluated with an xCELLigence system according to changes in the slopes of the curves, calculated from the Cell Index in the selected time interval. Cells treated with 10 µM **Z9** provided the positive control. Data are normalized to the control cells (in 0.1% DMSO), which show the defined 100% migration. Data are means ± standard deviation from two independent assays, each performed in duplicate or triplicate.

Table 1. Inhibitory activities against CatX of the triazole-benzodioxine derivatives. Values are means ± standard deviation of two independent experiments, each performed in duplicate.



Cpd	X	Y	R	Residual activity at 50 µM (%)
Z9		S		15 ± 2
7		S		23 ± 5
8		S		17 ± 2
9		S		24 ± 3

10		S		20 ± 4
11		S		35 ± 3
12		S		55 ± 7
13		S		22 ± 4
14		S		28 ± 5
15		S		30 ± 4
16		S		37 ± 5
17		S		43 ± 6
18		S		33 ± 1
19		S		17 ± 0
20		S		15 ± 1

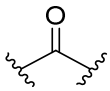
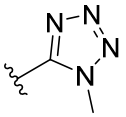
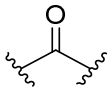
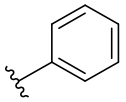
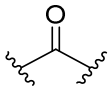
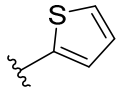
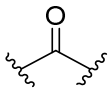
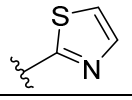
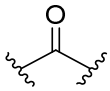
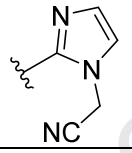
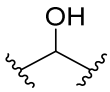
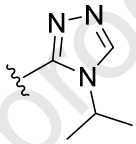
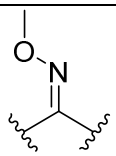
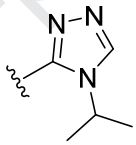
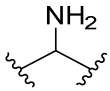
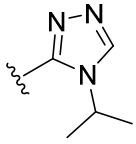
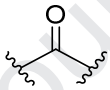
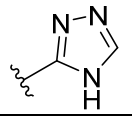
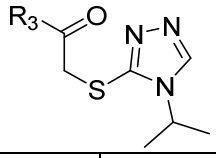
21		S		36 ± 0
22		S		48 ± 2
23		S		39 ± 1
24		S		22 ± 3
25		S		12 ± 3
26		S		98 ± 2
27		S		90 ± 0
28		S		90 ± 1
32		O		71 ± 5

Table 2. Inhibitory activities against CatX of compounds with various benzodioxine ring replacements. Values are means ± standard deviation of two independent experiments, each performed in duplicate.



Cpd	R_3	Residual activity at 50 µM (%)	Cpd	R_3	Residual activity at 50 µM (%)

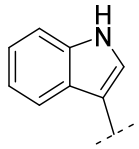
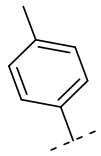
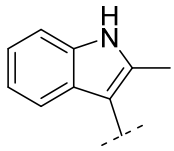
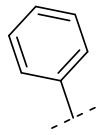
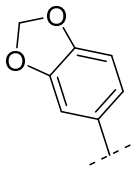
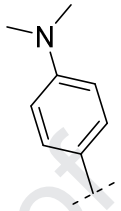
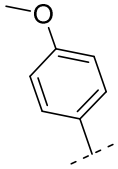
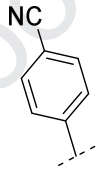
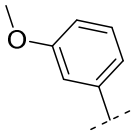
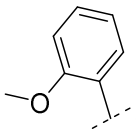
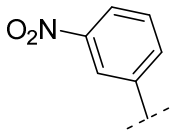
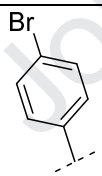
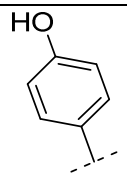
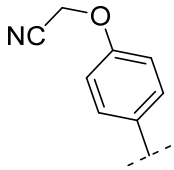
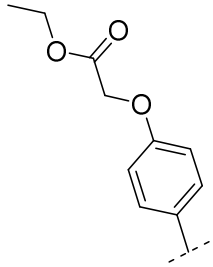
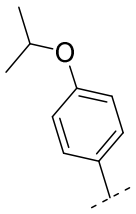
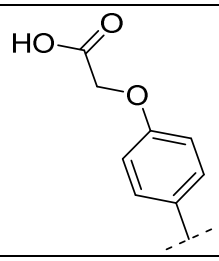
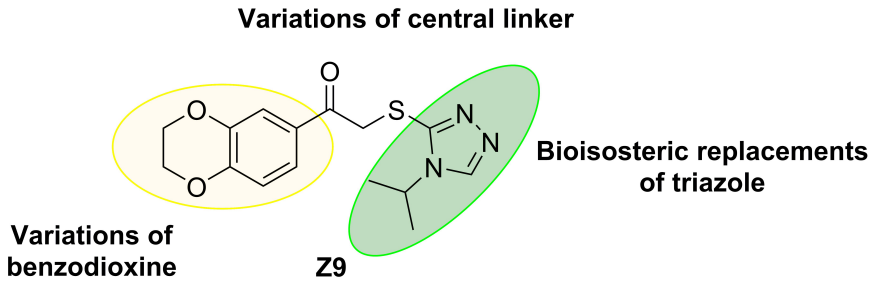
37		27 ± 4	**44**		75 ± 3
38		62 ± 6	**45**		100 ± 1
39		24 ± 1	**46**		41 ± 17
40		66 ± 30	**47**		92 ± 2
41		73 ± 5	**48**		80 ± 39
42		49 ± 0	**49**		100 ± 21
43		77 ± 1	**50**		73 ± 11
51		69 ± 6	**53**		60 ± 5
52		63 ± 4	**54**		85 ± 3

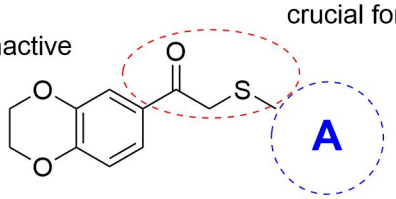
Table 3: IC_{50} values of selected inhibitors of CatX. Values are means ± standard deviation of two independent experiments, each performed in duplicate.

Compound	IC_{50} (µM)
Z9	11.5 ± 1.5
8	11.8 ± 1.1
10	12.4 ± 0.2
19	13.6 ± 0.8
20	9.54 ± 0.85
25	7.13 ± 0.47

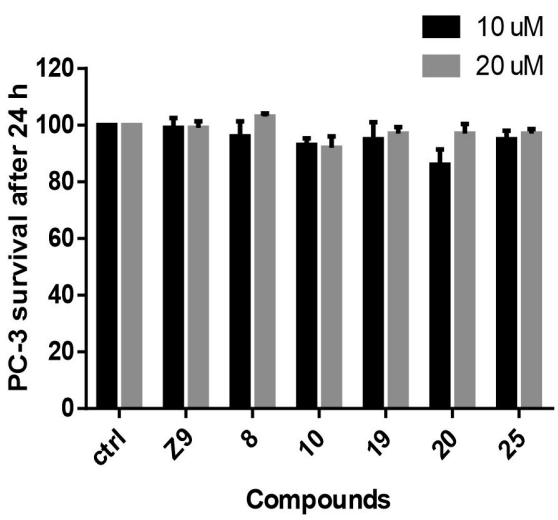


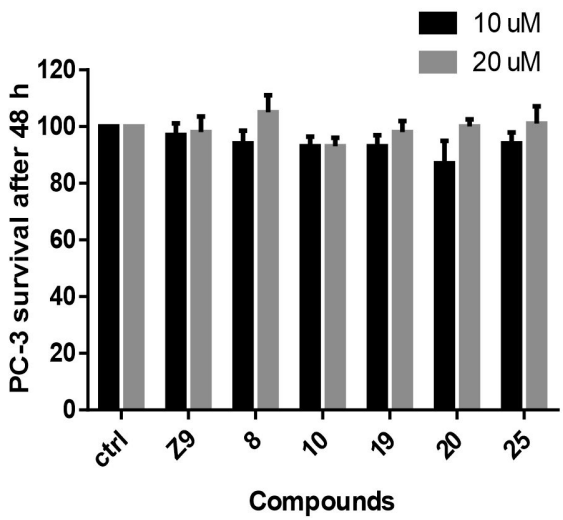
Variations of benzodioxine:
- indole: tolerated
- substituted phenyls: inactive

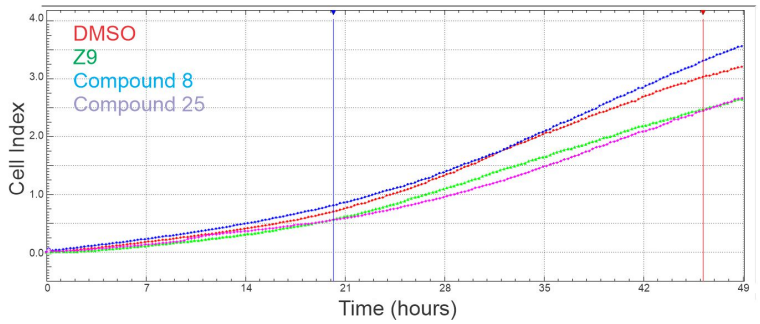
Ketomethylenethio linker:
crucial for inhibition

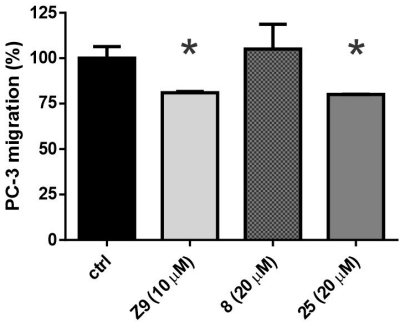


A: substituted/unsubstituted (hetero)cycles are well tollerated









Highlights

- Cathepsin X is up-regulated in cancer and neurodegenerative disorders.
- SARs exploration of the triazole-2,3-dihydrobenzo[*b*][1,4]dioxine inhibitors.
- Compound **25** inhibits PC-3 cell migration, which is under the control of CatX.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: