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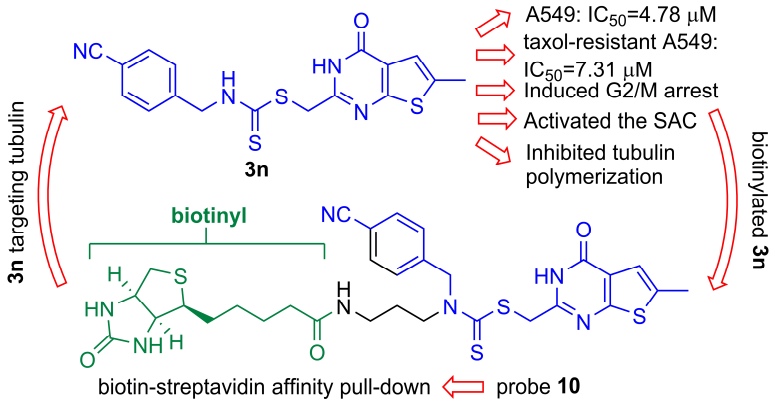

Synthesis, cytotoxic evaluation and target identification of thieno[2,3-*d*]pyrimidine derivatives with a dithiocarbamate side chain at C2 position

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ABSTRACT: Two series of thieno[2,3-*d*]pyrimidine derivatives bearing a dithiocarbamate side chain at the C2 position were synthesized and evaluated for cytotoxic activity in human lung cancer A549 and colon cancer HCT-116 cell lines. Compound **3n** exhibited the most cytotoxic effect on A549 cells with an IC_{50} value of 4.87 μM, inducing a cell cycle arrest at G2/M phase and activating the spindle assembly checkpoint (SAC). To identify the target protein(s) of **3n**, we incorporated biotin with **3n** through a three-carbon chain and an amide bond to synthesize probe **10**. The targeted proteins were pulled down from the A549 total cell lysate by biotin-streptavidin affinity purification and analyzed by mass spectrometry. Tubulin was the only protein identified, which is related to the SAC and directly binds to probe **10** both *in vivo* and *in vitro*. Furthermore, compound **3n** inhibited tubulin polymerization *in vitro* in a dose-dependent manner, competed with taxol in binding to tubulin, exerting cytotoxic

activity toward taxol-resistant A549 cells. These results demonstrate that thieno[2,3-*d*]pyrimidine derivative **3n** exhibits cytotoxicity in cancer cells by targeting tubulin to activate the SAC and potentially acts as a therapeutic lead compound for taxol-resistant cancers.

Keywords: Thieno[2,3-*d*]pyrimidine, dithiocarbamate, cytotoxicity, probe, target identification, tubulin.

Abbreviations: Boc, *tert*-butyloxycarbonyl; CPT, camptothecin; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulphoxide; DSB, DNA double-strand break; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; EDTA, ethylenediaminetetraacetic acid; 5-FU, 5-fluorouracil; γ-H2AX, histone H2AX phosphorylated on serine 139; pS10 H3, phospho-histone H3 on serine 10; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; PI, propidium iodide; rt, room temperature; SAC, spindle assembly checkpoint; SDS, Sodium dodecyl sulfate; TEA, triethylamine; TFA, trifluoroacetic acid.

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1. Introduction

Thieno[2,3-*d*]pyrimidine is an important heterocycle core structure from which various chemical derivatives can be synthesized. These derivatives have received notable attention over the past years due to the broad spectrum of biological activities, such as antitumor [1-3], antiviral [4], anti-inflammatory [5,6], analgesic [7] and antibacterial activity [8,9]. As such, many antitumor agents based on thieno[2,3-*d*]pyrimidine have been developed by researchers. Gangjee *et al*., [10,11] synthesized 2-aminothieno[2,3-*d*]pyrimidin-4(3*H*)-one derivatives bearing a benzoyl-L-glutamic acid moiety or lipophilic side chain at the 5-position and evaluated these derivatives as classical or non-classical antifolate antitumor agents. Of these compounds, one was an excellent dual inhibitor of human thymidylate synthase (TS, IC_{50} = 54 nM) and dihydrofolate reductase (DHFR, IC_{50} = 19 nM) and had nanomolar GI_{50} values in eight human cancer cell lines (Fig. 1; **I**) [11]. Bugge *et al*., designed and synthesized many thieno[2,3-*d*]pyrimidine derivatives and examined the effects of varying the 4-amino group and 6-aryl group on its inhibitory potency against epidermal growth factor receptor tyrosine kinase (EGFR-TK), antiproliferative and toxicity, and ADME properties. One of their compounds (Fig. 1; **II**) was a potential drug candidate for EGFR-driven cancers [12,13]. A high-throughput screening (HTS) assay revealed that tricycle tetrahydrobenzothieno[2,3-*d*]pyrimidine derivatives disrupt cell proliferation mediated by aberrant EGFR signaling. Wu *et al*. [14] introduced a chiral side chain and a Michael acceptor group into this scaffold and the resulting derivative showed >3 orders of magnitude enhanced HCC827 antiproliferative activity compared to the lead compound (Fig.1; **III**). This compound also inhibited gefitinib-resistant EGFR double mutant (DM, T790M/L858R) at nanomolar concentrations. Moreover, exposing a xenograft nude mouse model to this compound shrinked tumor growth significantly [14]. Although the majority of studies have focused on

substitutions at the C4, C5, and C6 positions of thieno[2,3-*d*]pyrimidine, there have been increasing reports of C2 substitutions on antitumor activity [15-17].

Natural and synthetic dithiocarbamates have excellent biological activities. Brassinin (Fig. 1) is a phytoalexin that can induce apoptosis in human prostate cancer cells by blocking the PI3K/Akt/mTOR/S6K1 signaling pathways [18]. Brassinin and its synthetic analogue 5-bromobrassinin exert anticancer effects by inhibiting indoleamine 2,3-dioxygenase, a pro-toleragenic enzyme that drives immune escape in cancer [19]. Li *et al*. [20] synthesized a compound with potent antiproliferative activity against four selected human cancer cell lines (HL-60, Bel-7402, SK-BR-3 and MDA-MB-468) (Fig. 1; **IV**). This compound induced apoptosis and G2/M cell-cycle arrest by disrupting spindle assembly during mitotic progression. We incorporated the dithiocarbamate moiety into the 6-position of 2-methylquinazlin-4(3*H*)-one to generate a set of compounds, several of which inhibited proliferation of human cancer cell lines with IC_{50} values at micromolar concentration (Fig. 1; **V** and **VI**) [21-23]. Recently, we transferred the dithiocarbamate moiety from the 6-position to 2-position of quinazlin-4(3*H*)-one to synthesize another series of compounds, most of which markedly inhibited the proliferation of A549, MCF-7, HeLa, HT29 and HCT-116 cell lines . In particular, one compound induced G2/M arrest in HT-29 cells by promoting tubulin polymerization *in vitro* and subsequently activating the spindle assembly checkpoint (Fig. 1; **VII**) [24]. The dithiocarbamate motif is also a suitable linkage region for combining different biologically active scaffolds and thus to design and synthesize new chemical entities [25].

< Fig. 1 >

In this study, compounds **3a–s** were synthesized as isosteric analogues of compound **VII**, in which the thieno[2,3-*d*]pyrimidin-4(3*H*)-one ring is connected to the sulfur atom of dithiocarbamate *via* a methylene group

(Scheme 1). For comparison purposes, we also synthesized compounds **6a–l** in which the thieno[2,3-*d*]pyrimidin-4(3*H*)-one ring is connected to the nitrogen atom of dithiocarbamate (Scheme 2). We evaluated the cytotoxic activity of the resulting compounds in cultured lung cancer A549 and colon HCT-116 cells. Of these compounds, **3n** was most potent towards A549 cells, which was carried forward to investigate the mechanisms underlying its cytotoxicity.

2. Results and discussion

*2.1. Synthesis of compounds **3a–s** and **6a–l***

Scheme 1 outlines the synthetic pathway of compounds **3a–s** in which the thieno[2,3-*d*]pyrimidin-4(3*H*)-one ring is connected to the sulfur atom of dithiocarbamate *via* a methylene group. Reacting ethyl 2-cyanoacetate and propionaldehyde with elemental sulfur in the presence of triethylamine (TEA) gave ethyl 2-amino-5-methylthiophene-3-carboxylate (intermediate **1**) [10]. This product was reacted with excess 2-chloroacetonitrile in the presence of hydrogen chloride gas to yield 2-(chloromethyl)-6-methylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (intermediate **2**) [26]. Intermediate **2** reacted with anhydrous potassium phosphate, carbon disulfide and the appropriate benzylamine, substituted benzylamine, or heterocyclylmethylamine to generate compounds **3a–s** (for R^1 groups, see Scheme 1).

< Scheme 1 >

Scheme 2 outlines the synthetic pathway of compounds **6a–l** in which the thieno[2,3-*d*]pyrimidin-4(3*H*)-one ring is linked to the nitrogen atom of dithiocarbamate. Reacting intermediate **2** with hexamethylenetetramine in tetrahydrofuran (THF) generated quaternary ammonium (**4**), which was treated with concentrated hydrochloric acid in methanol to give 2-(aminomethyl)-6-methylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (**5**), as previously

described [23]. Reaction of intermediate **5**, anhydrous potassium phosphate and carbon disulfide with the appropriate benzyl bromide, or substituted benzyl bromide, generated compounds **6a–l** (for R^2 groups, see Scheme 2).

< Scheme 2 >

*2.2. Synthesis of probe **10** (biotinylated compound **3n**) and control probe **14***

Cell proliferation assays indicated that compound **3n** had the most cytotoxic effect on A549 cells (IC_{50}, 4.87 μM) compared to all the other thieno[2,3-*d*]pyrimidine derivatives. To identify the target protein, we synthesized a biotinylated **3n** probe (**10**) for affinity pull-down assays. Here, 4-cyanobenzylbromide reacted with mono-protected propane-1,3-diamine to give intermediate **7** (Scheme 3). Reaction of **7** with 2-chloromethyl-6-methylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (**2**), carbon disulfide and potassium phosphate generated **8**, which was deprotected with trifluoroacetic acid (TFA) to give intermediate **9**. Finally, coupling **9** with D-(+)-biotin in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) yielded the probe (**10**).

< Scheme 3 >

We then prepared compound **11** by reacting intermediate **2** with 4-cyanobenzylamine, from which the carbodithionate moiety was removed (Scheme 4). This analogue of **3n** had no cytotoxicity towards A549 and HCT-116 cells, indicating that the carbodithionate moiety is critical to cytotoxic activity. Therefore, we introduced biotin into compound **11** *via* an amide bond and a three-carbon chain to synthesize compound **14**, which served as a control probe. Reaction of intermediate **2** with **7** gave compound **12** (Scheme 4). Removal of the Boc group in **12** with TFA, followed by the EDCI-mediated coupling of **13** with D-(+)-biotin produced compound **14**.

< Scheme 4 >

2.3. Structures of the target compounds

The structures of target compounds **3a–s** and **6a–l** were characterized by ^{1}H NMR, ^{13}C NMR and ESI-HRMS. In the ^{1}H NMR spectra of compounds **3a–s**, the signals of CH_3 groups at the C6 position of the thieno[2,3-*d*]pyrimidin-4(3*H*)-one ring appear at δ 2.48–2.50 ppm as doublets (*J* = 1.2 Hz), due to a long-range coupling of the C5-hydrogen to one of the methyl protons. Accordingly, the signals of C5-hydrogens appear at δ 7.05–7.07 ppm as doublets with the same *J* value (1.2 Hz). The singlets at δ 4.45–4.50 ppm are assigned to the CH_2 groups connected to the sulfur atoms, and the doublets at δ 4.65–4.99 ppm belong to the CH_2 groups linked to the NH groups in the dithiocarbamate moiety. The triplets at δ 10.29–10.70 ppm are assigned to the NH groups adjacent to the CH_2 groups, and the singlets at δ 12.50–12.54 ppm are attributed to the NH groups in the thieno[2,3-*d*]pyrimidin-4(3*H*)-one ring. In addition to the signals as discussed above, the signals ranging from δ 6.36 to 8.53 ppm belong to CH groups in phenyl, substituted phenyl or heterocyclic rings. The ^{13}C NMR spectra of compounds **3a–s** exhibit characteristic signals at δ 15.48–16.42 for methyl carbons, δ 36.89–37.83 and 43.62–50.87 for *S*-methylene and *N*-methylene carbons, δ 162.93–163.45 and 194.74–196.54 for carbonyl and thiocarbonyl carbons, and other expected signals as well. The ^{1}H NMR and ^{13}C NMR spectra of compounds **6a–l** are very similar to those of compounds **3a–s**. The ESI-HRMS data of all the target compounds is in good agreement with their calculated values.

The dithiocarbamate N–C(=S) single bonds (Fig. 2, shown in red) have partial double bond character. For compounds **3a–s** and **6a–l**, rotations around the N–C(=S) bonds are free due to a small hydrogen atom attached to each nitrogen atom of the dithiocarbamates; doubling of signals was not observed in their ^{1}H NMR spectra. In intermediates **8**, **9** and probe **10**, however, the hydrogen atom attached to the nitrogen atom of dithiocarbamate is substituted by a (*tert*-butyloxycarbonyl)aminopropyl (**8**), 3-aminopropyl (**9**) or biotinylaminopropyl group (**10**), respectively, and a 4-cyanobenzyl group is attached to the nitrogen atom. Rotations around the C–N(=S) bonds in these molecules (**8–10**) are restricted, and they can exist as rotamers (rotational isomers). The protons of

intermediates **8** and **9,** all exhibited two sets of peaks in the ^{1}H NMR spectra (in a nearly 3:2, or 1:1 ratio, respectively) corresponding to the two rotamers (Fig. 2). For probe **10**, protons (except those in the biotin moiety) also exhibited major and minor signals (in a nearly 3:2 ratio) in the ^{1}H NMR spectrum. As intermediate **8** or probe **10** has a more bulky *tert*-butyloxycarbonyl (Boc) or biotinyl group than a proton in **9**, the *E*-rotamers are more stable than the *Z*-rotamers, and the former accounts for the larger proportion in the mixture (~60%). The ^{13}C NMR spectrum of probe **10** showed a similar signal doubling pattern for the carbon atoms except for those in the biotin moiety and the methyl group at the C5-position of thieno[2,3-*d*]pyrimidine.

< Fig. 2 >

2.4. Cytotoxic activity

Compounds **3a**−**s** and **6a**−**l** were initially evaluated by MTS assay at a single concentration of 30 μM for their cytotoxicity towards A549 (lung cancer), HeLa (cervical carcinoma), MCF-7 and MDA-MB-231 (breast adenocarcinoma), HCT-116 and HT-29 (colorectal cancer) cells. Both A549 and HCT-116 cells were more sensitive than all other cells to compounds **3a**−**s**. However, hardly any cancer cells tested were sensitive to compounds **6a**−**l** at 30 μM (data not shown). These results indicated that connection of the thieno[2,3-*d*]pyrimidine ring to the sulfur atom of the dithiocarbamate moiety is essential for cytotoxicity.

The compounds in the **3a**−**s** series that induced ≥50% inhibition compared to vehicle were considered "active". We therefore analyzed the effects of these active compounds across a range of concentrations to determine the IC_{50} values for 50% inhibition of cellular proliferation (Tables 1). 5-Fluorouracil (5-FU) was used as a positive control. Most of the compounds **3a**−**s** exhibited a cytotoxic effect on cultured A549 and HCT-116 cells (IC_{50} < 30 μM), and they were generally more active in A549 than HCT-116 cells (Table 1). Compound **3a**

(without a substituent on the benzene ring) had an IC_{50} value of 29.53 μM against A549 cells. Compounds with a methyl (**3b**) or methoxy (**3c**) group at the 4-position of benzene ring were comparable to the parent compound **3a**, whereas compound **3d** (with a methoxy group at the 2-position of benzene ring) was more potent towards A549 cells than **3a**. Compound **3e** containing a 2,4-dimethoxy phenyl group exhibited weak cytotoxic activity. Compounds containing 3,4,5-trimethoxyphenyl (**3f**) or 3,4-methylenedioxyphenyl group (**3g**) were more active than compound **3a**. Compounds containing a moderate electron-withdrawing group at the 4-position of the benzene ring, namely, **3h** (4-bromo), **3i** (4-chloro) or **3k** (4-fluoro), but not **3l** (2-fluoro), were more cytotoxic than the parent compound **3a**. Compound **3j** (containing a 2,4-dichlorophenyl group) had equivalent activity as **3a**, whereas **3m** (containing a 2,4-difluorophenyl group) was more active than **3a**. Notably, compounds bearing a strong electron-withdrawing group at the 4-position of benzene ring, **3n** (4-cyano) and **3o** (4-nitro), were more potent than **3a**, with **3n** being the most potent against A549 cells (IC_{50}, 4.87 μM).

Replacement of the phenyl group with an electron-rich heterocycle in compound **3a** resulted in compounds **3p** (R^1 = thiophen-2-yl) and **3q** (R^1 = furan-2-yl), which were comparable to or less potent than **3a**, respectively. However, compounds containing an electron-deficient pyridinyl groups, namely, **3r** (R^1 = pyridin-3-yl) and **3s** (R^1 = pyridin-4-yl), were more potent than **3a**. These results demonstrate that aryl groups with an electron-withdrawing group instead of an electron-donating group are favorable for cytotoxicity, suggesting that the density of the electron cloud on aryl or heteroaryl groups is critical for cytotoxicity.

Most compounds in the **3a**−**s** series had IC_{50} values >20 μM in HCT-116 cells. Only four compounds, namely, **3d**, **3f**, **3r** and **3s**, had IC_{50} values <20 μM, but they were less potent than 5-FU. By contrast, compound **3n** had a comparable IC_{50} value to 5-FU (4.78 versus 3.52 μM) towards A549 cells. Therefore, compound **3n** was taken

forward to further investigate the mechanisms underlying cytotoxicity of cancer cells.

< Table 1 >

*2.5. Compound **3n** induced G2/M arrest in A549 cells*

We first examined the effects of compound **3n** on cell-cycle progression. A549 cells were exposed to **3n** at various concentrations (0.5×, 1.0×, or 2.0× the IC_{50}), 5-FU (IC_{50}), or taxol (0.10 μM) for 24 h, and analyzed for DNA content by flow cytometry. Three independent experiments were performed (Table S1 and Fig. 3A), and the results from one representative experiment are shown in Fig. 3B. In agreement with previous reports, A549 cells treated with 5-FU or taxol for 24 h arrested in S-phase or G2/M phase, respectively; we observed an obvious G2/M phase arrest in **3n**-treated A549 cells. Moreover, we observed a dose-dependent accumulation of G2/M cells when using concentrations from half to twice of the **3n** IC_{50} value.

< Fig. 3 >

*2.6. Compound **3n** did not induce DNA double strand breaks in A549 cells*

As compound **3n** induces G2/M arrest in A549 cells, we reasoned that **3n** might induce DNA damage. To test this hypothesis, we treated A549 cells with camptothecin (CPT, 2 μM) for 2 h or compound **3n** for 12 h. Immunofluorescence staining and immunoblotting using an antibody specific for the DSB (DNA double-strands break) marker γ-H2AX (histone H2AX phosphorylated on serine 139) was performed to examine whether these compounds could induce DSBs in cultured cancer cells. CPT is an inhibitor of topoisomerase I and induces replication-dependent DNA damage. Here, we found that treatment with CPT, but not compound **3n**, resulted in distinct γ-H2AX foci formation (Fig. 4A) and an increase in phosphorylation of H2AX and CHK1 (in response to DNA damage, ATR phosphorylates CHK1 at Ser345) (Fig. 4B). These results demonstrated that compound **3n**

cannot induce DNA damage under these conditions.

< Fig. 4 >

*2.7. Compound **3n** activated the spindle assembly checkpoint*

Both DNA damage-induced G2/M checkpoint activation and tubulin poison-induced spindle-assembly checkpoint activation can result in G2/M arrest. As compound **3n** failed to induce DNA damage, we speculated that compound **3n** might activate the spindle assembly checkpoint to elicit G2/M arrest. To this end, we determined the mitotic indexes of A549 cells treated with nocodazole or compound **3n** for different durations. The mitotic index was determined by the percentage of cells positive for phospho-histone H3 on serine 10 (pS10 H3). S10 phosphorylation starts at prophase and quickly disappears when cells move from metaphase to anaphase [27,28]. Therefore, cells in G2 or M phase can be distinguished by immunostaining with a pS10 H3 antibody, and the percentage of M phase cells can be determined by flow cytometry. Nocodazole elicits spindle-assembly checkpoint activation mainly through binding tubulin, which destabilizes tubulin polymerization and arrests cells in prometaphase. Nocodazole treatment led to a continuing increase of mitotic indexes from 2 to 16 hours (Table 2). Compound **3n** treatment resulted in a similar increase in the mitotic indexes, but to a less extent (Table 2, Fig. 5, A and B). Immunoblotting analysis confirmed that H3 phosphorylation at S10 increased following compound **3n** exposure from 12 h up to 24 h (Fig. 5C). BubR1 (MAD-3 like) protein is an essential kinase for kinetochores to establish microtubule attachments and the spindle assembly checkpoint. BubR1 is hyperphosphorylated by PLK1 during mitosis, which stabilizes the kinetochore attachments, leading to spindle checkpoint activation [29,30]. A549 cells treated with compound **3n** resulted in an increase of slow migration form of BubR1, which suggests a phosphorylation modification (Fig. 5C). Taken together, these results are consistent with compound **3n** activating

the spindle assembly checkpoint.

< Table 2 >

< Fig. 5 >

*2.8. Affinity purification and identification of compound **3n**-binding proteins*

To identify the target protein(s) of compound **3n**, we incubated probe **10** (biotinylated compound **3n**) with A549 total cell lysate. Streptavidin-coated magnetic beads were then used to pull down the biotinylated probe and associated binding proteins, which were resolved by SDS−PAGE and visualized by silver staining (Fig. 6). Both biotin alone (left lane) and the control probe (right lane) only pulled down a single band at the size of about 55 kDa, while probe **10** (middle lane) pulled down numerous bands. The binding proteins were eluted from the streptavidin-biotin probes and digested *in situ* with trypsin, the resulting peptide mixture was analyzed by mass spectrometry (Q Exactive HF LC/MS). The protein identities were determined by the molecular weight of the peptide mass fingerprint using pFind software (version 2.8, Chinese Academy of Sciences) searching from the UniProt database (Table 3). Among the major proteins pulled down by probe **10** (Table 3), both Tubulin α and Tubulin β functionally link to the SAC.

< Fig. 6 >

< Table 3 >

We then sought to determine if compound **3n** directly binds to tubulin. We found by immunoblotting analysis that α-tubulin was pulled down by the probe **10**, but not by biotin or the control probe **14** (Fig. 7A). As the pull-down of α-tubulin by the probe may be indirect, we carried out streptavidin-pulldown assays using purified porcine neuronal tubulin and the probes. It was found that α-tubulin was present in the protein complex pulled

down by probe **10,** but not by biotin or the control probe **14**, through immunoblotting analysis (Fig. 7B). These results indicate that α-tubulin can directly bind compound **3n**.

< Fig. 7 >

*2.9. Compound **3n** interferes with tubulin polymerization in vitro*

Given that compound **3n** directly binds tubulin, we sought to determine if the compound would interfere with tubulin function. *In vitro* tubulin polymerization assays were conducted with **3n** at various concentrations (5, 10 and 20 μM). Nocodazole (a well-known inhibitor of tubulin polymerization) and taxol (a stabilizer of tubulin polymerization) were used as controls. Here, we found that compound **3n** compromised tubulin polymerization in a dose-dependent manner, with less effect at 20 μM when compared to nocodazole treatment (Fig. 8).

< Fig. 8 >

*2.10. Compound **3n** competes with taxol in binding to tubulin*

The data thus far prove that compound **3n** targets tubulin, likely activating the SAC through destabilizing tubulin polymerization. There are three distinct and well-documented domains in tubulin for binding to paclitaxel, colchicine and vinblastine [31,32]. To determine if compound **3n** competitively binds to any of these three domains, we preincubated tubulin with excess taxol, colchicine, vinblastine, or nocodazole whose binding region in tubulin overlaps with that of colchicines [33], for 2 h and then repeated the binding experiment. We found that preincubation of tubulin with colchicine, vinblastine or nocodazole did not affect the binding of the probe with tubulin, while the probe bound less tubulin when preincubated with taxol (Fig. 9A, 9B). Next, we performed binding experiments using purified tubulin and probe **10**. The bead-bound probe-tubulin complex was then incubated with increasing concentrations of nocodazole, taxol, vinblastine, or colchicine before SDS-PAGE and

immunoblotting. Again, we found that the increasing doses of nocodazole, vinblastine, or colchicine did not reduce the amount of bound tubulin with the probe (Fig. 9D, 9E, 9F), while less tubulin bound the probe after incubation with taxol (Fig. 9C). These results demonstrated that compound **3n** competes with taxol in binding to tubulin.

< Fig. 9 >

Competitive binding assays uncovered that compound **3n** may share the same binding site as taxol in tubulin. However, in contrast to taxol stabilizing tubulin polymerization, **3n** inhibits tubulin polymerization. We therefore exposed taxol-resistant A549 cells to compound **3n** or taxol for 72 h, and then assessed cell proliferation by MTS assay. Taxol was ineffective in taxol-resistant A549 cells (IC_{50} > 30 μM), whereas compound **3n** markedly inhibited the proliferation of taxol-resistant A549 cells (IC_{50} = 7.31 ± 0.11 μM). Although **3n** was less potent to taxol-resistant A549 cells than A549 cells, it may be a potential lead for the development of anticancer agents treating taxol-resistant cancers.

3. Conclusion

This study designed and synthesized two series of compounds by incorporating a dithiocarbamate moiety into the C2 position of thieno[2,3-*d*]pyrimidine scaffold. These compounds (**3a**−**s** and **6a**−**l**) were evaluated as antitumor agents in cultured lung cancer A549 and colon HCT-116 cells. Most compounds in the series **3a**−**s** (in which thieno[2,3-*d*]pyrimidine is linked to the sulfur atom of dithiocarbamate) were cytotoxic towards A549 and HCT-116 cells. However, compounds **6a**−**l** (in which thieno[2,3-*d*]pyrimidine is linked to the nitrogen atom of dithiocarbamate) were inactive against the two cancer cell lines. Flow cytometry analysis demonstrated that the representive compound **3n** arrests A549 cells in G2/M phase and activates the spindle assembly checkpoint. *In*

vitro tubulin polymerization assays revealed that compound **3n** inhibited tubulin polymerization in a dose-dependent manner. LC-MS/MS mass spectrometry analysis and both *in vitro* and *in vivo* binding assays uncovered that tubulin directly binds to the biotinylated conjugating **3n** (probe **10**). Unexpectedly, compound **3n** competes with taxol, a stabilizer of tubulin polymerization, in binding to tubulin, and thus inhibits proliferation of taxol-resistant A549 cells. Taken together, thieno[2,3-*d*]pyrimidine derivatives bearing a dithiocarbamate side chain at the C2-position interfere with tubulin polymerization to activate the spindle assembly checkpoint, arrest the cell cycle at G2/M phase and exert cytotoxicity toward taxol-resistant A549 cells, potentially acting as a novel therapeutic lead compound for taxol-resistant cancers.

4. Experimental

4.1. Chemistry

Melting points were determined on an XT5B microscopic melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian VNMRS-600 spectrometer at 600 MHz using tetramethylsilane (TMS) as an internal standard. ^{13}C NMR spectra were recorded on a Varian VNMRS-600 spectrometer at 150 MHz using tetramethylsilane (TMS) as internal standard. High-resolution electrospray ionization (HR-ESI) mass spectra were recorded on a Thermo Scientific LTQ Orbitrap Discovery (Bremen, Germany) mass spectrometer. Column chromatography was carried out on a silica gel (200–300 mesh). High performance liquid chromatography (HPLC) analyses were performed on an Agilent 1200 Series HPLC instrument with a G1314B VWD (variable-wavelength detector) (λ = 256 nm, mobile phase: MeOH:H_2O = 7:3 (v/v)), and the data are presented in the Supplementary data. The purity of all compounds used in the biological studies was ≥ 95%. Arylmethylamines, heterocyclylmethylamines and arylmethylbromides were obtained commercially and used without further

purification.

*4.1.1. Preparation of ethyl 2-amino-5-methylthiophene-3-carboxylate (intermediate **1**)*

A mixture of ethyl cyanoacetate (2.26 g, 20 mmol), triethylamine (1.02 g, 10 mmol) and sulfur (0.64 g, 20 mmol) in *N*,*N*-dimethylformamide (DMF, 20 mL) was stirred at 50 °C for 30 min. Propionaldehyde (1.16 g, 20 mmol) was added dropwise, and the reaction mixture was stirred at room temperature for 2 h. The mixture was poured into ice-cold water and extracted three times with ethyl acetate (15 mL, each). The combined extracts were washed with brine, dried over anhydrous sodium sulfate, and filtered and concentrated *in vacuo*. The residue was purified by column chromatography on a silica gel (petroleum/ethyl acetate = 9:1) to afford intermediate **1** as a pale yellow solid (2.32 g, 63%), mp 46−47°C (Lit [10] mp 45−47 °C). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 1.22 (t, J = 7.2 Hz, 3H, CH_3CH$_2$), 2.18 (d, J = 0.6 Hz, 3H, CH$_3$), 4.14 (q, J = 7.2 Hz, 2H, CH$_3$CH_2), 6.49 (s, 1H, thiophene 5-H), 7.08 (s, 2H, NH$_2$). ESI-HRMS *m/z*: calcd for $C_8H_{12}NO_2S$ ([M+H]$^+$): 186.0589; found:186.0584.

*4.1.2. Preparation of 2-(chloromethyl)-6-methylthieno[2,3-d]pyrimidin-4(3H)-one (intermediate **2**)*

A solution of ethyl 2-amino-5-methylthiophene-3-carboxylate (**1**) (32.4 g, 175 mmol) and chloroacetonitrile (15.8 g, 210 mmol) in 1,4-dioxane (300 mL) was stirred at room temperature for 6 h, while hydrogen chloride gas was simultaneously passed through the solution. The resulting solid was collected by filtration and washed with 1,4-dioxane. The solid was suspended in water (50 mL) and the mixture was adjusted with 25% aqueous ammonia to pH 7. After stirring for 2 h, the precipitate was collected by filtration and air-dried, and purified by re-crystallization from 1,4-dioxane to give intermediate **2** as a white solid (32.3 g, 86%), mp 211−215 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.52 (d, J = 1.2 Hz, 3H, CH$_3$), 4.56 (s, 2H, ClCH$_2$), 7.11 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 12.74 (s, 1H, NH). ESI-HRMS *m/z*: calcd for $C_8H_8ClN_2OS$ ([M+H]$^+$): 215.0046;

found:215.0042.

*4.1.3. General preparation procedure for compounds **3a**−**s***

A suspension of amine (2 mmol), carbon disulfide (0.8 mL, 13.3 mmol), and finely powdered potassium phosphate (0.52 g, 2.45 mmol) in DMF (20 mL) was stirred at room temperature for 0.5 h. After adding 2-(chloromethyl)-6-methylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (**2**) (0.43 g, 2 mmol), stirring was continued overnight. The mixture was poured into water (100 mL) and the separated solid was collected by filtration and air-dried. The crude product was purified by column chromatography on a silica gel using the eluent indicated below to give compounds **3a**−**s**.

*4.1.3.1. (6-Methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl benzylcarbamodithioate (**3a**).*

Yield: 86%, white solid, mp 258−260 °C (eluent: dichloromethane/methanol = 98:2), HPLC retention time (rt) = 6.644 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (s, 3H, CH_3), 4.48 (s, 2H, SCH_2), 4.84 (d, J = 5.4 Hz, 2H, CH_2NH), 7.06 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.28 (m, 3H, Ph 2′-H, 4′-H, 6′-H), 7.34 (t, J = 7.2 Hz, 2H, Ph 3′-H, 5′-H), 10.58 (t, J = 5.4 Hz, 1H, CH_2N*H*), 12.52 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.95 (6-CH_3), 37.33 (SCH_2), 50.29 (CH_2NH), 119.42 (4a-C), 123.62 (5-C), 127.70 (4′-C), 128.08 (2C, 2′-C, 6′-C), 128.83 (2C, 3′-C, 5′-C), 137.39 (6-C), 137.42 (1′-C), 153.73 (7a-C), 157.83 (2-C), 163.44 (4-C), 195.52 (C=S). ESI-HRMS *m/z*: calcd for $C_{16}H_{16}N_3OS_3$ ($[M+H]^+$): 362.0455; found: 362.0442.

*4.1.3.2. (6-Methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl 4-methylbenzylcarbamodithioate (**3b**).*

Yield: 37%, light yellow solid, mp 168−171 °C (eluent: dichloromethane/methanol = 99:1), HPLC rt = 10.314 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.28 (s, 3H, 4′-CH_3), 2.49 (d, J = 1.2 Hz, 3H, 6-CH_3), 4.47 (s, 2H, SCH_2), 4.77 (d, J = 5.4 Hz, 2H, CH_2NH), 7.06 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.14 (d, J = 7.8

Hz, 2H, Ph 3′-H, 5′-H), 7.18 (d, *J* = 7.8 Hz, 2H, Ph 2′-H, 6′-H), 10.53 (t, *J* = 5.4 Hz, 1H, CH_2NH), 12.51 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.94 (6-CH_3), 21.15 (4′-CH_3), 37.33 (SCH_2), 50.12 (CH_2NH), 119.42 (4a-C), 123.62 (5-C), 128.13 (2C, 2′-C, 6′-C), 129.37 (2C, 3′-C, 5′-C), 134.35 (1′-C), 136.87 (4′-C), 137.39 (6-C), 153.75 (7a-C), 157.82 (2-C), 163.42 (4-C), 195.26 (C=S). ESI-HRMS *m/z*: calcd for $C_{17}H_{18}N_3OS_3$ ($[M+H]^+$): 376.0612; found: 376.0611.

*4.1.3.3. (6-Methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl 4-methoxybenzylcarbamodithioate (**3c**)*

Yield: 45%, light yellow solid, mp 195−198 °C (eluent: dichloromethane/methanol = 98:2), HPLC rt = 7.163 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, *J* = 1.2 Hz, 3H, CH_3), 3.73 (s, 3H, OCH_3), 4.47 (s, 2H, SCH_2), 4.75 (d, *J* = 5.4 Hz, 2H, CH_2NH), 6.89 (d, *J* = 8.4 Hz, 2H, Ph 3′-H, 5′-H), 7.06 (d, *J* = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.23 (d, *J* = 8.4 Hz, 2H, Ph 2′-H, 6′-H), 10.50 (t, *J* = 5.4 Hz, 1H, CH_2NH), 12.51 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.94 (6-CH_3), 37.32 (SCH_2), 49.90 (CH_2NH), 55.52 (4′-OCH_3), 114.24 (2C, 3′-C, 5′-C), 119.42 (4a-C), 123.62 (5-C), 129.29 (1′-C), 129.64 (2C, 2′-C, 6′-C), 137.39 (6-C), 153.77 (7a-C), 157.82 (2-C), 159.02 (4′-C), 163.40 (4-C), 195.02 (C=S). ESI-HRMS *m/z*: calcd for $C_{17}H_{18}N_3O_2S_3$ ($[M+H]^+$): 392.0561; found: 392.0562.

*4.1.3.4. (6-Methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl 2-methoxybenzylcarbamodithioate (**3d**)*

Yield: 47%, light yellow solid, mp 251−253 °C (eluent: dichloromethane/methanol = 99.5:0.5), HPLC rt = 8.382 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, *J* = 1.2 Hz, 3H, CH_3), 3.81 (s, 3H, OCH_3), 4.47 (s, 2H, SCH_2), 4.75 (d, *J* = 5.4 Hz, 2H, CH_2NH), 6.91 (t, *J* = 7.2 Hz, 1H, Ph 5′-H), 7.01 (d, *J* = 7.2 Hz, 1H, Ph 3′-H), 7.06 (d, *J* = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.17 (d, *J* = 7.2 Hz, 1H, Ph 6′-H), 7.28 (t, *J* = 7.2 Hz, 1H, Ph

4′-H), 10.40 (t, *J* = 5.4 Hz, 1H, CH_2NH), 12.52 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.95 (6-CH_3), 37.36 (SCH_2), 46.02 (CH_2NH), 55.83 (2′-OCH_3), 111.15 (3′-C), 119.43 (4a-C), 120.56 (5′-C), 123.63 (5-C), 124.60 (4′-C), 128.78 (6′-C), 129.15 (1′-C), 137.39 (6-C), 153.82 (7a-C), 157.29 (2′-C), 157.83 (2-C), 163.42 (4-C), 195.31 (C=S). ESI-HRMS *m/z*: calcd for $C_{17}H_{18}N_3O_2S_3$ ($[M+H]^+$): 392.0561; found:392.0545.

*4.1.3.5. (6-Methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl 2,4-dimethoxybenzylcarbamodithioate (**3e**)*

Yield: 61%, yellow solid, mp 170−173 °C (eluent: dichloromethane/methanol = 95:5), HPLC rt = 6.805 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, *J* = 1.2 Hz, 3H, CH_3), 3.75 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 4.45 (s, 2H, SCH_2), 4.65 (d, *J* = 5.4 Hz, 2H, CH_2NH), 6.47 (dd, *J* = 7.8, 2.4 Hz, 1H, Ph 5′-H), 6.57 (d, *J* = 2.4 Hz, 1H, Ph 3′-H), 7.06 (d, *J* = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.10 (d, *J* = 7.8 Hz, 1H, Ph 6′-H), 10.29 (t, *J* = 5.4 Hz, 1H, CH_2NH), 12.50 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.93 (6-CH_3), 37.35 (SCH_2), 45.95 (CH_2NH), 55.68 (OCH_3), 55.93 (OCH_3), 98.80 (3′-C), 104.87 (5′-C), 116.72 (1′-C), 119.42 (4a-C), 123.62 (5-C), 130.16 (6′-C), 137.39 (6-C), 153.90 (7a-C), 157.81 (2-C), 158.47 (4′-C), 160.69 (2′-C), 163.37 (4-C), 194.74 (C=S). ESI-HRMS *m/z*: calcd for $C_{18}H_{20}N_3O_3S_3$ ($[M+H]^+$): 422.0667; found: 422.0663.

*4.1.3.6. (6-Methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl 3,4,5-trimethoxybenzylcarbamodithioate (**3f**)*

Yield: 51%, light yellow solid, mp 193−195 °C (eluent: dichloromethane/methanol = 98:2 to 95 :5), HPLC rt = 4.342 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.48 (s, 3H, CH_3), 3.64 (s, 3H, OCH_3), 3.73 (s, 6H, $2OCH_3$), 4.49 (s, 2H, SCH_2), 4.75 (d, *J* = 5.4 Hz, 2H, CH_2NH), 6.44 (s, 2H, Ph 2′-H, 6′-H), 7.05 (s, 1H, thieno[2,3-*d*]pyrimidine 5-H), 10.50 (t, *J* = 5.4 Hz, 1H, CH_2NH), 12.51 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.91 (6-CH_3),

37.37 (SCH_2), 50.87 (CH_2NH), 56.25 (2C, 3′-OCH_3, 5′-OCH_3), 60.41 (4′-OCH_3), 105.84 (2C, 2′-C, 6′-C), 119.40 (4a-C), 123.60 (5-C), 132.79 (1′-C), 137.24 (4′-C), 137.38 (6-C), 153.27 (2C, 3′-C, 5′-C), 153.84 (7a-C), 157.81 (2-C), 163.37 (4-C), 195.34 (C=S). ESI-HRMS *m/z*: calcd for $C_{19}H_{22}N_3O_4S_3$ ($[M+H]^+$): 452.0772; found: 452.0770.

*4.1.3.7. (6-Methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl (3,4-methylenedioxy)benzylcarbamodithioate (**3g**)*

Yield: 33%, pale brown solid, mp 183−187 °C (eluent: dichloromethane/methanol = 98 :2 to 95 :5), HPLC rt = 5.478 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, J = 1.2 Hz, 3H, CH_3), 4.47 (s, 2H, SCH_2), 4.72 (d, J = 5.4 Hz, 2H, CH_2NH), 6.00 (s, 2H, OCH_2O), 6.79 (dd, J = 7.8, 1.8 Hz, 1H, Ph 6′-H), 6.86 (d, J = 7.8 Hz, 1H, Ph 5′-H), 6.87 (d, J = 1.8 Hz, 1H, Ph 2′-H), 7.05 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 10.49 (t, J = 5.4 Hz, 1H, CH_2N*H*), 12.51 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.48 (6-CH_3), 36.89 (SCH_2), 49.73 (CH_2NH), 100.94 (OCH_2O), 108.11 (2′-C), 108.39 (5′-C), 118.96 (4a-C), 121.25 (6′-C), 123.17 (5-C), 130.62 (1′-C), 136.96 (6-C), 146.49 (3′-C), 147.24 (4′-C), 153.30 (7a-C), 157.37 (2-C), 162.93 (4-C), 194.74 (C=S). ESI-HRMS *m/z*: calcd for $C_{17}H_{16}N_3O_3S_3$ ($[M+H]^+$): 406.0354; found: 406.0339.

*4.1.3.8. (6-Methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl 4-bromobenzylcarbamodithioate (**3h**)*

Yield: 31%, light yellow solid, mp 198−202 °C (eluent: dichloromethane/methanol = 97:3), HPLC rt = 12.545 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, J = 1.2 Hz, 3H, CH_3), 4.48 (s, 2H, SCH_2), 4.80 (d, J = 5.4 Hz, 2H, CH_2NH), 7.06 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.25 (d, J = 8.4 Hz, 2H, Ph 2′-H, 6′-H), 7.52 (d, J = 8.4 Hz, 2H, Ph 3′-H, 5′-H), 10.58 (t, J = 5.4 Hz, 1H, CH_2N*H*), 12.52 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.96 (6-CH_3), 37.36 (SCH_2), 49.53 (CH_2NH), 119.43 (4a-C), 120.79 (4′-C), 123.63 (5-C), 130.29

(2C, 2′-C, 6′-C), 131.70 (2C, 3′-C, 5′-C), 136.90 (1′-C), 137.40 (6-C), 153.65 (7a-C), 157.82 (2-C), 163.40 (4-C), 195.76 (C=S). ESI-HRMS *m/z*: calcd for $C_{16}H_{15}BrN_3OS_3$ ($[M+H]^+$): 439.9561, 441.9540; found: 439.9552, 441.9532.

*4.1.3.9. (6-Methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl 4-chlorobenzylcarbamodithioate (**3i**)*

Yield: 31%, light yellow solid, mp 163−166 °C (eluent: dichloromethane/methanol = 99:1 to 98 :2), HPLC rt = 10.519 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, J = 1.2 Hz, 3H, CH_3), 4.48 (s, 2H, SCH_2), 4.82 (d, J = 6.0 Hz, 2H, CH_2NH), 7.06 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.31 (d, J = 8.4 Hz, 2H, Ph 2′-H, 6′-H), 7.39 (d, J = 8.4 Hz, 2H, Ph 3′-H, 5′-H), 10.58 (t, J = 6.0 Hz, 1H, CH_2N*H*), 12.52 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 16.42 (6-CH_3), 37.83 (SCH_2), 49.96 (CH_2NH), 119.90 (4a-C), 124.10 (5-C), 129.26 (2C, 3′-C, 5′-C), 130.42 (2C, 2′-C, 6′-C), 132.77 (4′-C), 136.95 (1′-C), 137.88 (6-C), 154.13 (7a-C), 158.30 (2-C), 163.89 (4-C), 196.23 (C=S). ESI-HRMS *m/z*: calcd for $C_{16}H_{13}ClN_3OS_3$ ($[M+H]^+$): 396.0066; found: 396.0062.

*4.1.3.10. (6-Methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl 2,4-dichlorobenzylcarbamodithioate (**3j**)*

Yield: 14%, light yellow solid, mp 182−186 °C (eluent: dichloromethane/methanol = 98:2), HPLC rt = 19.537 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.50 (d, J = 1.2 Hz, 3H, CH_3), 4.49 (s, 2H, SCH_2), 4.83 (d, J = 5.4 Hz, 2H, CH_2NH), 7.06 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.32 (d, J = 8.4 Hz, 1H, Ph 6′-H), 7.41 (dd, J = 8.4, 2.4 Hz, 1H, Ph 5′-H), 7.64 (d, J = 2.4 Hz, Ph 3′-H), 10.56 (t, J = 5.4 Hz, 1H, CH_2N*H*), 12.53 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.95 (6-CH_3), 37.43 (SCH_2), 47.77 (CH_2NH), 119.44 (4a-C), 123.64 (5-C), 127.75 (5′-C), 129.22 (3′-C), 131.06 (6′-C), 133.22 (4′-C), 133.68 (1′-C), 133.76 (2′-C), 137.40 (6-C), 153.60 (7a-C), 157.82 (2-C), 163.40 (4-C), 196.26 (C=S). ESI-HRMS *m/z*: calcd for $C_{16}H_{14}Cl_2N_3OS_3$ ($[M+H]^+$):

429.9676, 431.9647; found: 429.9665, 431.9625.

*4.1.3.11. (6-Methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl 4-fluorobenzylcarbamodithioate (**3k**)*

Yield: 63%, light yellow solid, mp 155−158 °C (eluent: dichloromethane/methanol = 98:2), HPLC rt = 6.526 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, J = 1.2 Hz, 3H, CH_3), 4.48 (s, 2H, SCH_2), 4.81 (d, J = 5.4 Hz, 2H, CH_2NH), 7.06 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.16 (t, J = 9.0 Hz, 2H, Ph 3′-H, 5′-H), 7.34 (dd, J = 9.0, 5.4 Hz, 2H, Ph 2′-H, 6′-H), 10.57 (t, J = 5.4 Hz, 1H, CH_2N*H*), 12.52 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.93 (6-CH_3), 37.34 (SCH_2), 49.52 (CH_2NH), 115.58 (d, J = 21.3 Hz, 2C, 3′-C, 5′-C), 119.41 (4a-C), 123.62 (5-C), 130.20 (d, J = 8.25 Hz, 2C, 2′-C, 6′-C), 133.61 (d, J = 3.0 Hz, 1′-C), 137.39 (6-C), 153.68 (7a-C), 157.82 (2-C), 161.85 (d, J = 241.65 Hz, 4′-C), 163.41 (4-C), 195.55 (C=S). ESI-HRMS *m/z*: calcd for $C_{16}H_{15}FN_3OS_3$ ($[M+H]^+$): 380.0361; found: 380.0354.

*4.1.3.12. (6-Methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl 2-fluorobenzylcarbamodithioate (**3l**)*

Yield: 76%, yellow solid, mp 153−158 °C (eluent: dichloromethane/methanol = 98:2), HPLC rt = 6.285 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, J = 1.2 Hz, 3H, CH_3), 4.48 (s, 2H, SCH_2), 4.84 (d, J = 5.4 Hz, 2H, CH_2NH), 7.06 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.16-7.22 (m, 2H, Ph-H), 7.33-7.37 (m, 2H, Ph-H), 10.56 (t, J = 5.4 Hz, 1H, CH_2N*H*), 12.52 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.94 (6-CH_3), 37.38 (SCH_2), 44.35 (d, J = 4.5 Hz, CH_2NH), 115.70 (d, J = 21.15 Hz, 3′-C), 119.43 (4a-C), 123.63 (5-C), 124.03 (d, J = 14.55 Hz, 1′-C), 124.79 (d, J = 3.45 Hz, 5′-C), 129.95 (d, J = 8.1 Hz, 4′-C), 130.40 (d, J = 4.35 Hz, 6′-C), 137.39 (6-C), 153.69 (7a-C), 157.82 (2-C), 160.59 (d, J = 244.05 Hz, 2′-C), 163.43 (4-C), 195.88 (C=S). ESI-HRMS *m/z*: calcd for $C_{16}H_{15}FN_3OS_3$ ($[M+H]^+$): 380.0361; found: 380.0357.

*4.1.3.13. (6-Methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl 2,4-difluorobenzylcarbamodithioate (**3m**)*

Yield: 66%, pale brown solid, mp 153−158 °C (eluent: dichloromethane/methanol = 98:2), HPLC rt = 8.675 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (s, 3H, CH_3), 4.48 (s, 2H, SCH_2), 4.79 (d, J = 5.4 Hz, 2H, CH_2NH), 7.06 (s, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.07 (td, J = 8.4, 2.4 Hz, 1H, Ph 3′-H), 7.25 (td, J = 8.4, 2.4 Hz, 1H, Ph 5′-H), 7.41 (m, 1H, Ph 6′-H), 10.54 (t, J = 5.4 Hz, 1H, CH_2N*H*), 12.52 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.94 (6-CH_3), 37.38 (SCH_2), 43.96 (d, J = 3.0 Hz, CH_2NH), 104.31 (t, J = 25.65 Hz, 3′-C), 111.80 (dd, J = 21.15, 3.6 Hz, 5′-C), 119.42 (4a-C), 120.44 (dd, J = 15.0, 3.75 Hz, 1′-C), 123.62 (5-C), 131.79 (dd, J = 9.75, 5.7 Hz, 6′-C), 137.40 (6-C), 153.66 (7a-C), 157.81 (2-C), 160.69 (dd, J = 246.75, 12.3 Hz, 2′-C), 162.19 (dd, J = 244.65, 12.0 Hz, 4′-C), 163.41 (4-C), 195.87 (C=S). ESI-HRMS *m/z*: calcd for $C_{16}H_{14}F_2N_3OS_3$ ($[M+H]^+$): 398.0267; found: 398.0263.

*4.1.3.14. (6-Methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl 4-cyanobenzylcarbamodithioate (**3n**)*

Yield: 41%, light yellow solid, mp 195−198 °C (eluent: dichloromethane/methanol = 99.5:0.5 to 98:2), HPLC rt = 4.484 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.50 (d, J = 1.2 Hz, 3H, CH_3), 4.49 (s, 2H, SCH_2), 4.92 (d, J = 6.0 Hz, 2H, CH_2NH), 7.06 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.46 (d, J = 8.4 Hz, 2H, Ph 2′-H, 6′-H), 7.80 (d, J = 8.4 Hz, 2H, Ph 3′-H, 5′-H), 10.65 (t, J = 6.0 Hz, 1H, CH_2N*H*), 12.53 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.95 (6-CH_3), 37.39 (SCH_2), 49.68 (CH_2NH), 110.40 (4′-C), 119.21 (CN), 119.43 (4a-C), 123.63 (5-C), 128.73 (2C, 2′-C, 6′-C), 132.77 (2C, 3′-C, 5′-C), 137.42 (6-C), 143.34 (1′-C), 153.56 (7a-C), 157.83 (2-C), 163.42 (4-C), 196.33 (C=S). ESI-HRMS *m/z*: calcd for $C_{17}H_{15}N_4OS_3$ ($[M+H]^+$): 387.0408; found: 387.0398.

*4.1.3.15. (6-Methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl 4-nitrobenzylcarbamodithioate (**3o**)*

Yield: 46%, yellow solid, mp 183−188 °C (eluent: dichloromethane/methanol = 99:1 to 98:2) , HPLC rt = 5.650 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, J = 1.2 Hz, 3H, CH_3), 4.50 (s, 2H, SCH_2), 4.96 (d, J = 5.4 Hz, 2H, CH_2NH), 7.06 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.53 (d, J = 8.4 Hz, 2H, Ph 2′-H, 6′-H), 8.19 (d, J = 8.4 Hz, 2H, Ph 3′-H, 5′-H), 10.70 (t, J = 5.4 Hz, 1H, CH_2N*H*), 12.54 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.48 (6-CH_3), 36.96 (SCH_2), 48.99 (CH_2NH), 119.00 (4a-C), 123.18 (5-C), 123.53 (2C, 3′-C, 5′-C), 128.46 (2C, 2′-C, 6′-C), 136.94 (6-C), 145.04 (1′-C), 146.63 (4′-C), 153.12 (7a-C), 157.38 (2-C), 162.97 (4-C), 195.99 (C=S). ESI-HRMS *m/z*: calcd for $C_{16}H_{15}N_4O_3S_3$ ($[M+H]^+$): 407.0306; found: 437.0308.

*4.1.3.16. (6-Methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl (thiophen-2-ylmethyl)carbamodithioate (**3p**)*

Yield: 24%, off-white solid, mp 192−195 °C (eluent: dichloromethane/methanol = 97:3), HPLC rt = 5.617 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, J = 1.2 Hz, 3H, CH_3), 4.48 (s, 2H, SCH_2), 4.99 (d, J = 5.4 Hz, 2H, CH_2NH), 6.98 (dd, J = 5.4, 3.6 Hz, 1H, thiophene 4′-H), 7.05 (d, J = 1.2 Hz, thieno[2,3-*d*]pyrimidine 5-H), 7.07 (dd, J = 3.6, 1.2 Hz, 1H, thiophene 3′-H), 7.43 (dd, J = 4.8, 1.2 Hz, 1H, thiophene 5′-H), 10.62 (t, J = 5.4 Hz, 1H, CH_2N*H*), 12.51 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.94 (6-CH_3), 37.35 (SCH_2), 45.07 (CH_2NH), 119.40 (4a-C), 123.61 (5-C), 126.28 (3′-C), 127.04 (5′-C), 127.42 (4′-C), 137.39 (6-C), 139.48 (2′-C), 153.62 (7a-C), 157.81 (2-C), 163.42 (4-C), 195.36 (C=S). ESI-HRMS *m/z*: calcd for $C_{14}H_{14}N_3OS_4$ ($[M+H]^+$): 368.0020; found: 368.0018.

*4.1.3.17. (6-Methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl (furan-2-ylmethyl)carbamodithioate (**3q**)*

Yield: 25%, off-white solid, mp 181−184 °C (eluent: dichloromethane/methanol = 99:1 to 98:2), HPLC rt = 4.493 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, J = 1.2 Hz, 3H, CH_3), 4.47 (s, 2H, SCH_2), 4.80 (d, J = 4.8 Hz, 2H, CH_2NH), 6.36 (d, J = 3.0 Hz, 1H, furan 3′-H), 6.42 (dd, J = 3.0, 1.8 Hz, 1H, furan 4′-H), 7.06 (d, J = 1.2 Hz, thieno[2,3-*d*]pyrimidine 5-H), 7.62 (dd, J = 1.8, 0.6 Hz, 1H, furan 5′-H), 10.54 (t, J = 4.8 Hz, 1H, CH_2N*H*), 12.51 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.94 (6-CH_3), 37.36 (SCH_2), 43.62 (CH_2NH), 109.08 (3′-C), 111.00 (4′-C), 119.41 (4a-C), 123.61 (5-C), 137.39 (6-C), 143.12 (5′-C), 150.07 (2′-C), 153.71 (7a-C), 157.81 (2-C), 163.40 (4-C), 195.60 (C=S). ESI-HRMS *m/z*: calcd for $C_{14}H_{14}N_3O_2S_3$ ([M+H]$^+$): 352.0248; found: 352.0247.

*4.1.3.18. (6-Methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl (pyridin-3-ylmethyl)carbamodithioate (**3r**)*

Yield: 89%, white solid, mp 256−260 °C (eluent: dichloromethane/methanol = 90:10), HPLC rt = 2.656 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, J = 1.2 Hz, 3H, CH_3), 4.49 (s, 2H, SCH_2), 4.85 (s, 2H, CH_2NH), 7.06 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.37 (dd. J = 7.8, 4.8 Hz, 1H, pyridine 5′-H), 7.70 (dt, J = 7.8, 1.8 Hz, 1H, pyridine 4′-H), 8.49 (dd, J = 4.8, 1.2 Hz, 1H, pyridine 6′-H), 8.53 (d, J = 1.8 Hz, 1H, pyridine 2′-H), 10.62 (s, 1H, CH_2N*H*), 12.53 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.94 (6-CH_3), 37.37 (SCH_2), 47.88 (CH_2NH), 119.42 (4a-C), 123.62 (5-C), 123.95 (5′-C), 133.07 (3′-C), 135.91 (4′-C), 137.40 (6-C), 148.93 (6′-C), 149.53 (2′-C), 153.63 (7a-C), 157.82 (2-C), 163.42 (4-C), 195.92 (C=S). ESI-HRMS *m/z*: calcd for $C_{15}H_{15}N_4OS_3$ ([M+H]$^+$): 363.0408; found: 363.0404.

*4.1.3.19. (6-Methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl (pyridin-4-ylmethyl)carbamodithioate (**3s**)*

Yield: 58%, off-white solid, mp 260−263 °C (eluent: dichloromethane/methanol = 90:10), HPLC rt = 2.651 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.50 (s, 3H, CH_3), 4.50 (s, 2H, SCH_2), 4.87 (s, 2H, CH_2NH), 7.07 (d, J = 0.6 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.26 (d, J = 5.4 Hz, 2H, pyridine 3′-H, 5′-H), 8.51 (d, J = 5.4 Hz, 2H, pyridine 2′-H, 6′-H), 10.65 (s, 1H, CH_2N*H*), 12.54 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.90 (6-CH_3), 37.36 (SCH_2), 48.93 (CH_2NH), 119.43 (4a-C), 122.67 (2C, 3′-C, 5′-C), 123.62 (5-C), 137.41 (6-C), 146.46 (4′-C), 150.03 (2C, 2′-C, 6′-C), 153.58 (7a-C), 157.84 (2-C), 163.45 (4-C), 196.54 (C=S). ESI-HRMS *m/z*: calcd for $C_{15}H_{15}N_4OS_3$ ($[M+H]^+$): 363.0408; found: 363.0404.

*4.1.4. Preparation of 2-(aminomethyl)-6-methylthieno[2,3-d]pyrimidin-4(3H)-one (intermediate **5**)*

A solution of 2-(chloromethyl)-6-methylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (**2**) (4.3 g, 20 mmol) and hexamethylenetetramine (3.6 g, 26 mmol) in anhydrous tetrahydrofuran (THF, 100 mL) was stirred under reflux for 12 h. The mixture was allowed to cool to room temperature and the precipitate was collected by filtration, washed with 1,4-dioxane and air-dried to give intermediate **4** as an off-white solid, which was used directly in the next step without purification.

A mixture of intermediate **4** (6.98 g, 19.7 mmol), concentrated hydrochloric acid (50 mL) and methanol (50 mL) was stirred under reflux for 8 h. After cooling to room temperature, the seperated solid was collected by filtration, and washed with methanol. The solid was suspended in water and the mixture was adjusted with 25% aqueous ammonia to pH 10. The precipitate was collected by filtration and washed with a small volume of cold water. The air-dried crude product was purified by column chromatography on a silica gel (eluent:

dichloromethane/methanol = 8:2) to give intermediate **5** as a pale brown solid (1.5 g, 38%, overall yield of two steps), mp 223−228 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.51 (s, 3H, CH_3), 4.08 (s, 2H, CH_2), 7.11 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 9.58 (br s, 2H, NH_2). ESI-HRMS *m/z*: calcd for $C_8H_{10}N_3OS$ ($[M+H]^+$): 196.0545; found:196.0542.

*4.1.5. General preparation procedure for compounds **6a−l***

A suspension of 2-(aminomethyl)-6-methylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (**5**) (2 mmol), carbon disulfide (0.8 mL, 13.3 mmol), and finely powdered potassium phosphate (0.52 g, 2.45 mmol) in DMF (20 mL) was stirred at room temperature for 0.5 h. After adding the appropriate benzyl bromide (2 mmol), the mixture was stirred at room temperature overnight. The mixture was poured into water (20 mL) and the resulting precipitate was collected by filtration and air-dried. The crude product was purified by column chromatography on a silica gel using the eluent indicated below to give compounds **6a**−**l**.

*4.1.5.1. Benzyl ((6-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl)carbamodithioate (**6a**)*

Yield: 35%, white solid, mp 223−225 °C (eluent: dichloromethane/methanol = 98:2), HPLC rt = 8.827 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.50 (s, 3H, CH_3), 4.51 (s, 2H, SCH_2), 4.75 (d, J = 4.8 Hz, 2H, CH_2NH), 7.06 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.26 (t, J = 7.2 Hz, 1H, Ph 4′-H), 7.32 (t, J = 7.2 Hz, 2H, Ph 3′-H, 5′-H), 7.38 (d, J = 7.2 Hz, 2H, Ph 2′-H, 6′-H) 10.42 (t, J = 4.8 Hz, 1H, CH_2N*H*), 12.51 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.95 (6-CH_3), 38.98 (CH_2S), 48.32 (NHCH_2), 119.42 (4a-C), 123.77 (5-C), 127.67 (4′-C), 128.89 (2C, 3′-C, 5′-C), 129.37 (2C, 2′-C, 6′-C), 137.13 (1′-C), 137.41 (6-C), 153.39 (7a-C), 157.90 (2-C), 163.57 (4-C), 198.13 (C=S). ESI-HRMS *m/z*: calcd for $C_{16}H_{16}N_3OS_3$ ($[M+H]^+$): 362.0455; found: 362.0456.

*4.1.5.2. 4-Methylbenzyl ((6-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl)carbamodithioate (**6b**)*

Yield: 43%, white solid, mp 190−192 °C (eluent: dichloromethane/methanol = 98:2), HPLC rt = 13.388 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.27 (s, 3H, 4′-CH_3), 2.49 (d, J = 1.2 Hz, 3H, 6-CH_3), 4.46 (s, 2H, SCH_2), 4.75 (d, J = 5.4 Hz, 2H, CH_2NH), 7.06 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.12 (d, J = 7.8 Hz, 2H, Ph 3′-H, 5′-H), 7.26 (d, J = 7.8 Hz, 2H, Ph 2′-H, 6′-H), 10.38 (t, J = 5.4 Hz, 1H, CH_2N*H*), 12.50 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.94 (6-CH_3), 21.14 (4′-CH_3), 38.83 (CH_2S), 48.28 (NHCH_2), 119.42 (4a-C), 123.77 (5-C), 129.30 (2C, 3′-C, 5′-C), 129.45 (2C, 2′-C, 6′-C), 134.12 (1′-C), 136.88 (4′-C), 137.12 (6-C), 153.40 (7a-C), 157.89 (2-C), 163.56 (4-C), 198.21 (C=S). ESI-HRMS *m/z*: calcd for $C_{17}H_{18}N_3OS_3$ ($[M+H]^+$): 376.0612; found: 376.0613.

*4.1.5.3. 4-Methoxybenzyl ((6-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl)carbamodithioate (**6c**)*

Yield: 39%, white solid, mp 209−212 °C (eluent: dichloromethane/methanol = 99.5:0.5 to 98:2), HPLC rt = 7.163 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, J = 1.2 Hz, 3H, CH_3), 3.73 (s, 3H, OCH_3), 4.44 (s, 2H, SCH_2), 4.74 (d, J = 5.4 Hz, 2H, CH_2NH), 6.88 (d, J = 8.4 Hz, 2H, Ph 3′-H, 5′-H), 7.06 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.30 (d, J = 8.4 Hz, 2H, Ph 2′-H, 6′-H), 10.36 (t, J = 5.4 Hz, 1H, CH_2N*H*), 12.50 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.94 (6-CH_3), 38.62 (CH_2S), 48.24 (NHCH_2), 55.51 (4′-OCH_3), 114.32 (2C, 3′-C, 5′-C), 119.42 (4a-C), 123.78 (5-C), 128.92 (1′-C), 130.60 (2C, 2′-C, 6′-C), 137.12 (6-C), 153.42 (7a-C), 157.90 (2-C), 158.93 (4′-C), 163.57 (4-C), 198.29 (C=S). ESI-HRMS *m/z*: calcd for $C_{17}H_{18}N_3OS_3$ ($[M+H]^+$): 392.0561; found: 392.0561.

4.1.5.4. 3-Methoxybenzyl ((6-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl)carbamodithioate

(6d)

Yield: 33%, white solid, mp 204−205 °C (eluent: dichloromethane/methanol = 98:2), HPLC rt = 8.752 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, J = 1.2 Hz, 3H, CH_3), 3.73 (s, 3H, OCH_3), 4.48 (s, 2H, SCH_2), 4.75 (d, J = 5.4 Hz, 2H, CH_2NH), 6.83 (dt, J = 7.8, 1.8 Hz, 1H, Ph 4′-H), 6.95 (d, J = 7.8 Hz, 1H, Ph 6′-H) , 6.96 (d, J = 1.8 Hz, 1H, Ph 2′-H), 7.06 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.23 (t, J = 7.8 Hz, 1H, Ph 5′-H), 10.42 (t, J = 5.4 Hz, 1H, CH_2N*H*), 12.51 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.95 (6-CH_3), 38.99 (CH_2S), 48.34 (NHCH_2), 55.45 (3′-OCH_3), 113.17 (2′-C), 114.98 (4′-C), 119.42 (4a-C), 121.56 (6′-C), 123.78 (5-C), 129.96 (5′-C), 137.13 (6-C), 138.86 (1′-C), 153.40 (7a-C), 157.90 (2-C), 159.68 (3′-C), 163.58 (4-C), 198.15 (C=S). ESI-HRMS *m/z*: calcd for $C_{17}H_{18}N_3OS_3$ ([M+H]$^+$): 392.0561; found: 392.0564.

4.1.5.5. 2-Methoxybenzyl ((6-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl)carbamodithioate

(6e)

Yield: 35%, white solid, mp 210−213°C (eluent: dichloromethane/methanol = 99.5:0.5 to 98:2), HPLC rt = 8.783 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, J = 1.2 Hz, 3H, CH_3), 3.82 (s, 3H, OCH_3), 4.43 (s, 2H, SCH_2), 4.74 (d, J = 5.4 Hz, 2H, CH_2NH), 6.89 (td, J = 7.8, 0.6 Hz, 1H, Ph 5′-H), 7.01 (d, J = 8.4 Hz, 1H, Ph 3′-H), 7.06 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.28 (td, J = 7.8, 1.8 Hz, 1H, Ph 4′-H), 7.36 (dd, J = 7.2, 1.8 Hz, 1H, Ph 6′-H), 10.31 (t, J = 5.4 Hz, 1H, CH_2N*H*), 12.50 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.94 (6-CH_3), 34.26 (CH_2S), 48.21 (NHCH_2), 55.95 (2′-OCH_3), 111.39 (3′-C), 119.41 (4a-C), 120.73 (5′-C), 123.76 (5-C), 124.55 (1′-C), 129.46 (4′-C), 130.77 (6′-C), 137.11 (6-C), 153.46 (7a-C), 157.57 (2′-C), 157.90 (2-C), 163.57 (4-C), 198.63 (C=S). ESI-HRMS *m/z*: calcd for $C_{17}H_{18}N_3OS_3$ ([M+H]$^+$): 392.0561; found: 392.0556.

*4.1.5.6. 4-Fluorobenzyl ((6-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl)carbamodithioate (**6f**)*

Yield: 15%, white solid, mp 160−163 °C (eluent: dichloromethane/methanol = 98:2), HPLC rt = 9.802 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, J = 1.2 Hz, 3H, CH_3), 4.51 (s, 2H, SCH_2), 4.75 (d, J = 4.8 Hz, 2H, CH_2NH), 7.06 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.14 (t, J = 8.4 Hz, 2H, Ph 3′-H, 5′-H), 7.43 (dd, J = 8.4, 5.4 Hz, 2H, Ph 2′-H, 6′-H), 10.44 (t, J = 4.8 Hz, 1H, CH_2N*H*), 12.51 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.95 (6-CH_3), 38.06 (CH_2S), 48.35 (NHCH_2), 115.64 (d, J = 21.3 Hz, 2C, 3′-C, 5′-C), 119.42 (4a-C), 123.78 (5-C), 131.32 (d, J = 8.1 Hz, 2C, 2′-C, 6′-C), 133.84 (d, J = 2.85 Hz, 1′-C), 137.14 (6-C), 153.36 (7a-C), 157.90 (2-C), 161.76 (d, J = 241.95 Hz, 4′-C), 163.56 (4-C), 197.97 (C=S). ESI-HRMS *m/z*: calcd for $C_{16}H_{15}FN_3OS_3$ ($[M+H]^+$): 380.0361; found: 380.0357.

*4.1.5.7. 2,4-Difluorobenzyl ((6-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl)carbamodithioate (**6g**)*

Yield: 36%, white solid, mp 156−158 °C (eluent: dichloromethane/methanol = 98:2), HPLC rt = 11.881 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, J = 1.2 Hz, 3H, CH_3), 4.50 (s, 2H, SCH_2), 4.74 (d, J = 5.4 Hz, 2H, CH_2NH), 7.05 (m, 1H, Ph 3′-H), 7.06 (d, J = 2.4 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.26 (td, J = 9.6, 2.4 Hz, 1H, Ph 5′-H), 7.55 (dt, J = 8.4, 6.6 Hz, 1H, Ph 6′-H), 10.49 (t, J = 5.4 Hz, 1H, CH_2N*H*), 12.51 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.93 (6-CH_3), 31.97 (CH_2S), 48.36 (NHCH_2), 104.37 (t, J = 25.8 Hz, 3′-C), 111.93 (dd, J = 21.15, 3.6 Hz, 5′-C), 119.41 (4a-C), 120.74 (dd, J = 14.85, 3.75 Hz, 1′-C), 123.77 (5-C), 132.69 (dd, J = 9.75, 5.25 Hz, 6′-C), 137.14 (6-C), 153.30 (7a-C), 157.89 (2-C), 160.76 (dd, J = 247.2, 12.3 Hz, 2′-C), 162.11 (dd, J = 245.05, 12.15 Hz, 4′-C), 163.53 (4-C), 197.36 (C=S). ESI-HRMS *m/z*: calcd for $C_{16}H_{14}F_2N_3OS_3$ ($[M+H]^+$): 398.0267; found: 398.0267.

*4.1.5.8. 4-Chlorobenzyl ((6-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl)carbamodithioate (**6h**)*

Yield: 35%, white solid, mp 159−160 °C (eluent: dichloromethane/methanol = 98:2), HPLC rt = 12.623 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, J = 1.2 Hz, 3H, CH_3), 4.51 (s, 2H, SCH_2), 4.75 (d, J = 3.0 Hz, 2H, CH_2NH), 7.06 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.37 (d, J = 8.4 Hz, 2H, Ph 2′-H, 6′-H), 7.41 (d, J = 8.4 Hz, 2H, Ph 3′-H, 5′-H), 10.46 (t, J = 4.2 Hz, 1H, CH_2N*H*), 12.51 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.95 (6-CH_3), 38.04 (CH_2S), 48.40 (NHCH_2), 119.43 (4a-C), 123.78 (5-C), 128.80 (2C, 3′-C, 5′-C), 131.19 (2C, 2′-C, 6′-C), 132.24 (4′-C), 136.86 (1′-C), 137.14 (6-C), 153.33 (7a-C), 157.90 (2-C), 163.55 (4-C), 197.83 (C=S). ESI-HRMS *m/z*: calcd for $C_{16}H_{13}ClN_3OS_3$ ([M+H]$^+$): 396.0066; found: 396.0065.

*4.1.5.9. 4-Bromobenzyl ((6-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl)carbamodithioate (**6i**)*

Yield: 45%, white solid, mp 172−177 °C (eluent: dichloromethane/methanol = 97:3), HPLC rt = 19.535 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, J = 1.2 Hz, 3H, CH_3), 4.50 (s, 2H, SCH_2), 4.74 (d, J = 5.4 Hz, 2H, CH_2NH), 7.06 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.34 (d, J = 8.4 Hz, 2H, Ph 2′-H, 6′-H), 7.50 (d, J = 8.4 Hz, 2H, Ph 3′-H, 5′-H), 10.46 (t, J = 5.4 Hz, 1H, CH_2N*H*), 12.51 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-*d6*) δ: 15.95 (6-CH_3), 38.09 (CH_2S), 48.40 (NHCH_2), 119.42 (4a-C), 120.74 (4′-C), 123.78 (5-C), 131.54 (2C, 2′-C, 6′-C), 131.72 (2C, 3′-C, 5′-C), 137.14 (6-C), 137.29 (1′-C), 153.33 (7a-C), 157.89 (2-C), 163.55 (4-C), 197.80 (C=S). ESI-HRMS *m/z*: calcd for $C_{16}H_{15}BrN_3OS_3$ ([M+H]$^+$): 439.9561; found: 439.9560.

*4.1.5.10. 4-Nitrobenzyl ((6-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl)carbamodithioate (**6j**)*

Yield: 42%, white solid, mp 175−177 °C (eluent: dichloromethane/methanol = 98:2), HPLC rt = 7.032 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.49 (d, J = 1.2 Hz, 3H, CH_3), 4.67 (s, 2H, SCH_2), 4.75 (d, J = 5.4 Hz, 2H, CH_2NH), 7.06 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.65 (d, J = 8.4 Hz, 2H, Ph 2′-H, 6′-H), 8.18 (d, J

= 8.4 Hz, 2H, Ph 3′-H, 5′-H), 10.55 (t, *J* = 5.4 Hz, 1H, CH_2NH), 12.52 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.93 (6-CH_3), 37.95 (CH_2S), 48.54 ($NHCH_2$), 119.42 (4a-C), 123.79 (5-C), 123.91 (2C, 3′-C, 5′-C), 130.52 (2C, 2′-C, 6′-C), 137.14 (6-C), 146.29 (1′-C), 146.95 (4′-C), 153.26 (7a-C), 157.88 (2-C), 163.53 (4-C), 197.34 (C=S). ESI-HRMS *m/z*: calcd for $C_{16}H_{15}N_4O_3S_3$ ($[M+H]^+$): 407.0306; found: 407.0307.

*4.1.5.11. 4-Cyanobenzyl ((6-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl)carbamodithioate (**6k**)*

Yield: 31%, white solid, mp 161−164 °C (eluent: dichloromethane/methanol = 98:2), HPLC rt = 4.735 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 2.50 (d, *J* = 1.2 Hz, 3H, CH_3), 4.62 (s, 2H, SCH_2), 4.75 (d, *J* = 5.4 Hz, 2H, CH_2NH), 7.06 (d, *J* = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.58 (d, *J* = 8.4 Hz, 2H, Ph 2′-H, 6′-H), 7.78 (d, *J* = 8.4 Hz, 2H, Ph 3′-H, 5′-H), 10.53 (t, *J* = 5.4 Hz, 1H, CH_2NH), 12.52 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.94 (6-CH_3), 38.25 (CH_2S), 48.50 ($NHCH_2$), 110.32 (4′-C), 119.19 (CN), 119.41 (4a-C), 123.78 (5-C), 130.29 (2C, 2′-C, 6′-C), 132.71 (2C, 3′-C, 5′-C), 137.15 (6-C), 144.04 (1′-C), 153.26 (7a-C), 157.88 (2-C), 163.53 (4-C), 197.47 (C=S). ESI-HRMS *m/z*: calcd for $C_{17}H_{15}N_4OS_3$ ($[M+H]^+$): 387.0408; found:387.0410.

*4.1.5.12. 4-(Ethoxycarbonyl)benzyl ((6-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl)carbamodithioate (**6l**)*

Yield: 31%, white solid, mp 151−154 °C (eluent: dichloromethane/methanol = 99.5:0.5 to 98 :2), HPLC rt = 10.417 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 1.32 (t, *J* = 7.2 Hz, 3H, CH_3CH_2), 2.49 (d, *J* = 1.2 Hz, 3H, CH_3), 4.31 (q, *J* = 7.2 Hz, 2H, CH_3CH_2), 4.60 (s, 2H, SCH_2), 4.75 (d, *J* = 5.4 Hz, 2H, CH_2NH), 7.06 (d, *J* = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.52 (d, *J* = 7.8 Hz, 2H, Ph 2′-H, 6′-H), 7.90 (d, *J* = 7.8 Hz, 2H, Ph 3′-H, 5′-H), 10.49 (t, *J* = 5.4 Hz, 1H, CH_2NH), 12.52 (s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 14.60 (CH_2CH_3), 15.93

(6-CH_3), 38.43 (CH_2S), 48.43 ($NHCH_2$), 61.11 (CH_2CH_3), 119.43 (4a-C), 123.78 (5-C), 129.14 (4′-C), 129.61 (2C, 3′-C, 5′-C), 129.66 (2C, 2′-C, 6′-C), 137.11 (6-C), 143.42 (1′-C), 153.32 (7a-C), 157.89 (2-C), 163.55 (4-C), 165.88 (OC=O), 197.67 (C=S). ESI-HRMS *m/z*: calcd for $C_{19}H_{20}N_3O_3S_3$ ($[M+H]^+$): 434.0667; found: 434.0666.

*4.1.6. Preparation of tert-butyl (3-((4-cyanobenzyl)amino)propyl)carbamate (intermediate **7**)*

A solution of 4-cyanobenzylamine (1.0 g, 5.1 mmol) in ethanol (10 mL) was added dropwise to a solution of *tert*-butyl (3-aminopropyl)carbamate (2.6 g, 15 mmol) in ethanol (10 mL), and the mixture was stirred under reflux for 18 h. The solvent was removed by rotary evaporation and the residue was purified by column chromatography on a silica gel (eluent: dichloromethane/methanol/$NH_2{\cdot}H_2O$ = 98:2:0.5) to give intermediate **7** as a white solid (1.3 g, 85%), mp 58−60 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 1.36 (s, 9H, $(CH_3)_3CO$), 1.52 (m, 2H, $CH_2CH_2CH_2$), 2.34 (br s, 1H, CH_2NHCH_2), 2.44 (t, J = 7.2 Hz, 2H, $NHCH_2CH_2$), 2.96 (m, 2H, CH_2NHCO), 3.74 (s, 2H, $PhCH_2NH$), 6.77 (t, J = 5.4 Hz, 2H, CH_2NHCO), 7.53 (d, J = 7.8 Hz, 2H, Ph 2-H, 6-H), 7.77 (d, J = 7.8 Hz, 2H, Ph 3-H, 5-H). ESI-HRMS *m/z*: calcd for $C_{16}H_{24}N_3O_2$ ([M+H]+): 290.1863; found: 290.1865

*4.1.7. Preparation of (6-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl (3-(N-tert-butyloxycarbonyl)aminopropyl)(4-cyanobenzyl)carbamodithioate (intermediate **8**)*

A suspension of *tert*-butyl (3-((4-cyanobenzyl)amino)propyl)carbamate (**7**) (0.29 g, 1 mmol), carbon disulfide (0.5 mL, 8 mmol), and finely powdered potassium phosphate (0.25 g, 1.2 mmol) in dry DMF (10 mL) was stirred at room temperature for 0.5 h. After adding 2-(chloromethyl)-6-methylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (intermediate **2**) (0.19 g, 1 mmol), stirring was continued for 4 h. The mixture was poured into water (40 mL) and the precipitate was collected by filtration and air-dried. The crude product was purified by column chromatography on a silica gel (dichloromethane/methanol =

9:1) to give intermediate **8** as a pale yellow solid (0.3 g, 60%), mp 167−169 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 1.31, 1.34 (2s, 9H, $(CH_3)_3CO$), 1.77, 1.81 (2m, 2H, $CH_2CH_2CH_2$), 2.49, 2.50 (2s, 3H, CH_3), 2.92, 2.97 (2m, 2H, $NHCH_2CH_2$), 3.77, 3.92 (2t, J = 7.8 Hz, 2H, CH_2CH_2N), 4.51, 4.53 (2s, 2H, SCH_2), 5.19, 5.35 (2s, 2H, NCH_2Ph), 6.81, 6.92 (2d, J = 5.4 Hz, 1H, $NHCH_2$), 7.05, 7.07 (2s, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.40, 7.44 (2d, J = 8.4 Hz, 2H, Ph 2′-H, 6′-H), 7.81, 7.87 (2d, J = 8.4 Hz, 2H, Ph 3′-H, 5′-H), 12.54, 12.59 (2s, 1H, NH). ESI-HRMS *m/z*: calcd for $C_{25}H_{29}N_5O_3S_3Na$ ($[M+Na]^+$): 566.1325; found: 566.1330.

*4.1.8. Preparation of (6-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl (3-aminopropyl)(4-cyanobenzyl)carbamodithioate (intermediate **9**)*

Trifluoroacetic acid (TFA, 5 mL) was added dropwise to a solution of intermediate **8** (0.54 g, 1 mmol) in dichloromethane (10 mL). The mixture was stirred at room temperature for 10 min. The solvent and excess TFA was removed by rotary evaporation, and the residue was dissolved in an appropriate amount of methanol. The solution was cooled in an ice bath and adjusted with 25% aqueous ammonia to pH 9−10. After concentration under reduced pressure, the residue was subjected to column chromatography on a silica gel (eluent: dichloromethane/methanol = 9:1) to give intermediate **9** as a white solid (0.36 g, 80%), mp 188−190 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 1.95, 1.99 (2m, 2H, $CH_2CH_2CH_2$), 2.49, 2.50 (2s, 3H, CH_3), 2.80, 2.88 (2br s, 2H, $NH_2CH_2CH_2$), 3.88, 4.00 (2t, J = 7.2 Hz, 2H, CH_2CH_2N), 4.52, 4.56 (2s, 2H, SCH_2), 5.20, 5.34 (2s, 2H, NCH_2Ph), 7.05, 7.08 (2s, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.40, 7.45 (2d, J = 8.4 Hz, 2H, Ph 2′-H, 6′-H), 7.74, 7.80 (2br s, 2H, NH_2), 7.83, 7.89 (2d, J = 8.4 Hz, 2H, Ph 3′-H, 5′-H), 12.57, 12.64 (2s, 1H, NH). ESI-HRMS *m/z*: calcd for $C_{20}H_{22}N_5OS_3$ ($[M+H]^+$): 444.0981; found: 444.0987.

*4.1.9. Preparation of (6-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidin-2-yl)methyl (4-cyanobenzyl)(3-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)propyl)carba modithioate (probe **10**)*

A mixture of intermediate **9** (0.13 g, 0.3 mmol), D-(+)-biotin (73 mg, 0.3 mmol), EDCI (0.29 g, 1.5 mmol) and triethylamine (0.18 mg, 1.8 mmol) in DMF (6 mL) was stirred at room temperature for 12 h before being poured into ice-cold water (20 mL). The separated solid was collected by filtration and air-dried. The crude product was purified by column chromatography on a silica gel (eluent: dichloromethane/methanol = 98:2) to give probe **10** as a white solid (0.05 g, 25%), mp 125−127 °C, HPLC rt = 3.764 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 1.26 (m, 2H, biotin SCHCH$_2$CH_2), 1.45 (m, 3H, biotin SCHC*H*H, CH_2CH$_2$CO), 1.58 (m, 1H, biotin SCHCH*H*), 1.78, 1.83 (2m, 2H, NHCH$_2$CH_2CH$_2$N), 2.03 (t, *J* = 7.2 Hz, 2H, CH$_2$CH_2CO), 2.49, 2.50 (2s, 3H, CH$_3$), 2.57 (d, *J* = 12.6 Hz, 1H, biotin SCH*H*), 2.80 (dt, *J* = 12.6, 4.8 Hz, 1H, biotin SC*H*H), 3.05 (m, 1H, biotin SCH), 3.05, 3.09 (2m, 2H, NHCH_2CH$_2$), 3.78, 3.93 (2t, *J* = 7.8 Hz, 2H, CH$_2$CH_2N), 4.10 (m, 1H, biotin NHC*H*), 4.29 (m, 1H, biotin NHC*H*), 4.51, 4.54 (2s, 2H, SCH$_2$), 5.19, 5.35 (2s, 2H, NCH$_2$Ph), 6.36 (s, 1H, biotin CONH), 6.41 (s, 1H, biotin CONH), 7.05, 7.07 (2s, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.40, 7.44 (2d, *J* = 7.8 Hz, 2H, Ph 2′-H, 6′-H), 7.77, 7.87 (t, *J* = 6.0 Hz, 1H, CON*H*CH$_2$), 7.81, 7.88 (2d, *J* = 7.8 Hz, 2H, Ph 3′-H, 5′-H), 12.55, 12.60 (2s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.52 (6-CH$_3$), 25.27 (biotin SCHCH$_2$*C*H$_2$), 26.27 (biotin CH_2CH$_2$CO), 27.44 (biotin SCH*C*H$_2$CH$_2$), 28.01 (2C, CONHCH$_2$*C*H$_2$CH$_2$N, CH$_2$S), 35.22 (biotin CH$_2$*C*H$_2$CO), 35.76 (CONH*C*H$_2$CH$_2$CH$_2$N), 39.84 (biotin SCH$_2$), 50.85 (CONHCH$_2$CH$_2$*C*H$_2$N), 55.41 (biotin S*C*HCH$_2$), 56.78 (PhCH$_2$N), 59.19 (biotin NH*C*HCH), 61.02 (biotin NH*C*HCH$_2$), 110.02 (4′-C), 118.71 (CN), 119.00 (4a-C), 123.20 (5-C), 127.82 (2C, 2′-C, 6′-C), 132.40 (2C, 3′-C, 5′-C), 136.98 (6-C), 141.76 (1′-C), 152.40 (7a-C),

157.38 (2-C), 162.68 (biotin C=O), 163.08 (CH_2*C*ONHCH_2), 172.11 (4-C), 196.02 (C=S); additional signals for the minor rotamer: 28.18 (SCH_2), 28.20 (CONHCH$_2$*C*H$_2$CH$_2$N), 35.93 (CONH*C*H$_2$CH$_2$CH$_2$N), 53.53 (CONHCH$_2$CH$_2$*C*H$_2$N), 54.98 (PhCH$_2$N), 110.38 (4′-C), 118.63 (CN), 118.98 (4a-C), 123.11 (5-C), 127.53 (2C, 2′-C, 6′-C), 132.64 (2C, 3′-C, 5′-C), 136.90 (6-C), 141.27 (1′-C), 152.53 (7a-C), 157.35 (2-C), 171.97 (4-C), 195.33 (C=S). ESI-HRMS *m/z*: calcd for $C_{30}H_{36}N_7O_3S_4$ ($[M+H]^+$): 670.1757; found: 670.1762.

*4.1.10. Preparation of 2-((4-cyanobenzyl)amino)methyl-6-methylthieno[2,3-d]pyrimidin-4(3H)-one (intermediate **11**)*

2-(Chloromethyl)-6-methylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (**2**) (0.22 g, 1 mmol) was added to a solution of 4-cyanobenzylamine (0.26 g, 2 mmol) in dry DMF (10 mL), in a few aliquots. The mixture was stirred at room temperature for 24 h before being poured into ice-cold water (20 mL). The separated solid was collected by filtration and air-dried. The crude product was purified by column chromatography on a silica gel (eluent: dichloromethane/methanol = 95:5) to give compound **11** as a white solid (0.21 g, 70%), mp 189−190 °C, HPLC rt = 7.794 min. ^{1}H NMR (600 MHz, DMSO-d_6) *δ*: 2.49 (d, *J* = 1.2 Hz, 3H, CH_3), 3.65 (s, 2H, NCH_2), 3.81 (s, 2H, NCH_2), 7.05 (d, *J* = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.54 (d, *J* = 8.4 Hz, 2H, Ph 2′-H, 6′-H), 7.76 (d, *J* = 8.4 Hz, 2H, Ph 3′-H, 5′-H). ^{13}C NMR (150 MHz, DMSO-d_6) *δ:* 15.92, 50.94, 51.86, 109.79, 119.36, 119.40, 123.64, 129.19 (2C), 132.45 (2C), 136.87, 146.84, 156.65, 157.99, 163.66. ESI-HRMS *m/z*: calcd for $C_{16}H_{15}N_4OS$ ($[M+H]^+$): 311.0961; found: 311.0963.

*4.1.11. Preparation of 2-((3-(N-boc-amino)propyl)(4-cyanobenzyl)amino)methyl-6-methylthieno[2,3-d]pyrimidin-4(3H)-one (intermediate **12**)*

2-(Chloromethyl)-6-methylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (**2**) (0.19 g, 1 mmol) was added in a few

aliquots to a solution of intermediate **7** (0.29 g, 1 mmol) in dry DMF (10 mL). The reaction mixture was stirred at room temperature for 4 h. The mixture was poured into ice-cold water (15 mL) and extracted three times with dichloromethane (20 mL, each). The combined extracts were washed with brine and dried over anhydrous sodium sulfate. The solvent was removed by rotary evaporation, and the residue was purified by column chromatography on a silica gel (eluent: dichloromethane/methanol = 94:6, v/v) to give intermediate **12** as a pale yellow solid (0.39 g, 82%), mp 66−67 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 1.33 (s, 9H, $(CH_3)_3CO$), 1.56 (m, 2H, $CH_2CH_2CH_2$), 2.48 (d, J = 1.2 Hz, 3H, CH_3), 2.49 (m, 2H, CH_2CH_2N, overlapped with DMSO), 2.91 (m, 2H, $NHCH_2CH_2$), 3.60 (s, 2H, NCH_2), 3.76 (s, 2H, NCH_2), 6.72 (t, J = 6.0 Hz, 1H, $NHCH_2$), 7.03 (d, J = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.55 (d, J = 8.4 Hz, 2H, Ph 2′-H, 6′-H), 7.72 (d, J = 8.4 Hz, 2H, Ph 3′-H, 5′-H), 12.06 (s, 1H, NH). ESI-HRMS *m/z*: calcd for $C_{24}H_{30}N_5O_3S$ ($[M+H]^+$): 468.2064; found: 468.2069.

*4.1.12. Preparation of 2-((3-aminopropyl)(4-cyanobenzyl)amino)methyl-6-methylthieno[2,3-d]pyrimidin-4(3H)-one (intermediate **13**)*

Trifluoroacetic acid (5 mL) was added dropwise to an ice-bath cooled solution of intermediate **12** (0. 47 g, 1 mmol) in dichloromethane (10 mL). The mixture was stirred at room temperature for 1 h. After the removal of solvent by rotary evaporation, the residue was dissolved in methanol, and the solution was adjusted with 25% aqueous ammonia to pH 9–10. The solution was evaporated to dryness under reduced pressure, and the residue was subjected to column chromatography on a silica gel (eluent: dichloromethane/methanol = 9:1) to give intermediate **13** as a white solid (0.32 g, 86%), mp 67−69 °C. ^{1}H NMR (600 MHz, DMSO-d_6): 1.75 (m, 2H, $CH_2CH_2CH_2$), 2.46 (s, 3H, CH_3), 2.60 (t, J = 6.6 Hz, 2H, CH_2CH_2N), 2.80 (t, J = 6.6 Hz, 2H, NH_2CH_2), 3.54 (s, 2H, NCH_2), 3.76 (s, 2H, NCH_2), 6.97 (s, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.55 (d, J = 8.4 Hz, 2H, Ph 2′-H, 6′-H),

7.76 (d, *J* = 8.4 Hz, 2H, Ph 3′-H, 5′-H). ESI-HRMS *m/z*: calcd for $C_{19}H_{22}N_5OS$ ($[M+H]^+$): 368.1540; found: 368.1545.

*4.1.13. Preparation of 2-(N-((4-cyanobenzyl))(3-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)propyl)amino)methyl-6-methylthieno[2,3-d]pyrimidin-4(3H)-one (control probe **14**)*

A solution of intermediate **13** (0.24 g, 0.67 mmol), D-(+)-biotin (73 mg, 0.3 mmol), EDCI (0.29 mg, 1.5 mmol) and triethylamine (0.18 mg, 1.8 mmol) in DMF (6 mL) was stirred at room temperature for 24 h. The mixture was poured into ice-cold water (20 mL), and the solution was extracted three times with dichloromethane (20 mL, each). The combined extracts were washed with brine and dried over anhydrous sodium sulfate. The solvent was removed by rotary evaporation, and the residue was purified by column chromatography on a silica gel (eluent: dichloromethane/methanol = 95:5) to give probe **14** as a white solid (0.15 g, 68%), mp 125−126 °C, HPLC rt = 3.238 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 1.26 (m, 2H, biotin SCHCH$_2$C*H*$_2$), 1.45 (m, 3H, biotin SCHCH*H*, C*H*$_2$CH$_2$CONH), 1.58 (m, 3H, biotin SCHCH*H*, NHCH$_2$C*H*$_2$CH$_2$N), 1.99 (t, *J* = 7.2 Hz, 2H, CH$_2$C*H*$_2$CONH), 2.49 (d, *J* = 1.2 Hz, 3H, CH$_3$), 2.52 (m, 2H, CH$_2$C*H*$_2$N, overlapped with DMSO), 2.57 (d, *J* = 12.0 Hz, 1H, biotin SCH*H*), 2.80 (dd, *J* = 12.0, 5.4 Hz, 1H, biotin SC*H*H), 3.03 (m, 2H, NHC*H*$_2$CH$_2$), 3.07 (m, 1H, biotin SCH), 3.61 (s, 2H, NCH$_2$), 3.77 (s, 2H, NCH$_2$), 4.11 (m, 1 H, biotin NHC*H*), 4.30 (m, 1 H, biotin NHC*H*), 6.36 (s, 1H, biotin CONH), 6.41 (s, 1H, biotin CONH), 7.03 (d, *J* = 1.2 Hz, 1H, thieno[2,3-*d*]pyrimidine 5-H), 7.54 (d, *J* = 8.4 Hz, 2H, Ph 2′-H, 6′-H), 7.70 (t, *J* = 6.0 Hz, 1H, CON*H*CH$_2$), 7.72 (d, *J* = 8.4 Hz, 2H, Ph 3′-H, 5′-H), 12.08 (br s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 15.50 (6-CH$_3$), 25.30 (biotin SCHCH$_2$*C*H$_2$), 26.39 (biotin C*H*$_2$CH$_2$CO), 28.02 (biotin SCH*C*H$_2$CH$_2$), 28.23 (CONHCH$_2$*C*H$_2$CH$_2$N), 35.24 (biotin CH$_2$*C*H$_2$CO), 36.48 (CONH*C*H$_2$CH$_2$CH$_2$N), 39.84 (biotin CH$_2$S), 51.58 (CONHCH$_2$CH$_2$*C*H$_2$N), 55.43 (biotin S*C*HCH$_2$),

56.56 ($PhCH_2N$), 57.44 (NCH_2), 59.19 (biotin NH*C*HCH), 61.04 (biotin NH*C*HCH_2), 109.49 (4′-C), 118.88 (CN), 118.91 (4a-C), 123.37 (5-C), 129.45 (2C, 2′-C, 6′-C), 131.88 (2C, 3′-C, 5′-C), 136.67 (6-C), 145.01 (1′-C), 154.88 (7a-C), 157.51 (2-C), 162.69 (biotin C=O), 162.97 (CH_2*C*O$NHCH_2$), 171.84 (4-C). ESI-HRMS *m/z*: calcd for $C_{29}H_{36}N_7O_3S_2$ ($[M+H]^+$): 594.2316; found: 594.2319.

4.2. Biological assays

4.2.1. Cell Lines and culture conditions

A549 or HCT-116 cells were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco modified Eagle medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, PAN) and standard antibiotics (Hyclone), at 37 °C in a 5% CO_2 incubator. Taxol-resistant A549 cells were obtained from the Shanghai Institute for Biological Science (China).

4.2.2. Cell proliferation assay

A cell proliferation assay was performed as described [34]. Cell viability was tested using a CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega), based on the use of MTS. The data were obtained from triplicate wells in three independent experiments.

4.2.3. Determination of cell-cycle distribution in A549 cells and mitotic indexes

Cell-cycle profiles were determined by propidium iodide staining and fluorescence-activated cell sorting. The mitotic index was determined as described previously [35] using phospho-histone H3 (Ser10) rabbit polyclonal antibodies (dilution 1:1000, A301-844A, Bethyl Laboratories, Inc.).

4.2.4. Immunoblotting

Immunoblotting was performed as previously described [36]. Briefly, protein samples were separated on a

12% SDS−polyacrylmide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking, the membrane was incubated in 0.05% PBST containing 5% bovine serum albumin (BSA) for 30 min at room temperature and incubated with primary antibody specific to α-tubulin (1:2000, T6199, Sigma) overnight at 4 °C. The membrane was then washed with PBST, incubated with HRP-conjugated anti-mouse IgG (1:1000, 211-032-171, Jackson ImmunoResearch) secondary antibody at room temperature for 1 h. The immunoblots were visualized by ImageQuant™ LAS 4000 (GE Healthcare Life Sciences).

4.2.5. Biotin-streptavidin pull-down assay

For the pull-down endogenous tubulin assay, A549 cells were harvested and washed twice with ice-cold PBS, and then lysed in 600 μL NETN buffer (20 Mm Tris-HCl (pH 8.0), 0.15 M NaCl, 1 mM EDTA, 0.5% NP-40 and protease inhibitor cocktail) at 4°C. The cell lysate was incubated with equal volumes of biotin, probe **10** or control probe **14** at 4°C for 4 h. The mixtures were further incubated with Dynabeads (Dynabeads™ M-280 Streptavidin, Thermo Fisher Scientific Inc.) at 4°C for 1 h. The tubes were placed in a magnet for 2 min and the supernatant was discarded. The beads were then washed six times with NETN to remove unbound proteins. Finally, 120 μL of SDS sample buffer (1 M Tris (pH 6.8), Glyceral, 10% SDS, 0.5% Bromophenol, β-ME, H_2O) was added to each sample. For the *in vitro* tubulin-binding assay, porcine neuronal tubulin (catalogue # BK006P, Cytoskeleton, Inc.) was incubated with biotin, probe **10** or control probe **14** in NETN buffer in a total volume of 600 μL for 4 h at 4°C. The mixtures were incubated with Dynabeads for 1 h at 4 °C. The beads were washed with 1 mL NETN buffer six times. Finally, 120 μL of SDS sample buffer was added to each sample, followed by SDS-PAGE and immunoblotting.

4.2.6. Tubulin binding competition assay

For the competition assay, taxol, vinblastine, colchicine and nocodazole were preincubated with porcine neuronal tubulin at 4°C for 1 h before incubation with probe **10**. The mixtures were incubated with Dynabeads for another 1 h followed by washing with NETN buffer, as described in the pull-down assays.

We also performed a preincubation with porcine neuronal tubulin, probe **10** and Dynabeads together. After six washes with NETN buffer, 600 μL fresh NETN buffer was added, followed by incubation with increasing amounts of taxol, colchicine, vinblastine or nocodazole. After extensive washing with NETN buffer, 120 μL of SDS sample buffer was added to each sample, followed by SDS−PAGE and immunoblotting.

4.2.7. Silver stain assay

For the silver stain assay, the proteins binding biotin and probe **10** were released from the beads by boiling with SDS sample buffer at 100 °C for 5 min. The protein samples were then separated on a 10% SDS−polyacrylamide gel. The gel was visualized using a silver stain kit (Real-Times (Beijing) Biotechnology Co., Ltd, RTD6401).

*4.2.8. Identification of compound **3n** binding proteins with mass spectrometry*

Target-based complexes were prepared from the pull-down assay. The complexes were incubated at 65°C in 10 mM EDTA (pH 8.2) with 95% formamide to dissociate the proteins. The proteins were then precipitated with ice-cold acetone and resuspended in 8 M urea, 100 mM Tris (pH 8.5). After trypsin digestion, LC-MS/MS analysis was performed on an Easy-nLC 1000 UHPLC (Thermo Fisher Scientific) coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). Peptides were loaded on a pre-column (C18, 5 cm, ID 75 μm, 1.8 μm) and separated on an analytical column (C18, 13 cm, ID 75 μm, 3 μm). The gradient elution profile consisted

of 30% of mobile phase B for 80 min, then brought to 80% of mobile phase B over 20 min. The total run time was 100 min. Mobile phase A was 0.1% (v/v) formic acid in HPLC-grade doubly distilled H_2O, and mobile phase B was 0.1% (v/v) formic acid in HPLC-grade acetonitrile. The intense precursor ions from each full scan (resolution 120,000) were isolated for HCD MS2 (resolution 15,000; NCE 30) with a dynamic exclusion time of 60 sec. Data analysis was performed using pFind (version 2.8, Chinese Academy of Sciences).

4.2.9. Tubulin polymerization assay

In vitro tubulin polymerization assays were performed according to the manufacturer's instructions (Cytoskeleton, catalogue#BK006P). Polymerizations were monitored by an increase in absorbance at 340 nm over a 60 min period at 37 °C. The optical density at 340 nm was determined using a SpectraMax M5 spectrometer (Molecular Devices).

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Figure captions

Fig. 1. Chemical structures of antitumor thieno[2,3-*d*]pyrimidine derivatives (**I–III**), and natural and synthetic dithiocarbamates (Brassinin and compounds **IV–VII**).

Scheme 1. Synthetic route to compounds **3a–s**. Reagents and conditions: (a) S_8, TEA, DMF, 50 °C, 30 min; then, room temperature (rt) 2 h, 64%. (b) i. $ClCH_2CN$, HCl (g), 1,4-dioxane, rt, 6 h; ii. $NH_3{\cdot}H_2O$, 83%. (c) CS_2, K_3PO_4, R^1NH_2, DMF, rt, overnight.

Scheme 2. Synthetic route to compounds **6a–l**. Reagents and conditions: (a) Hexamethylenetetramine, THF, reflux, 12 h. (b) i. hydrochloric acid, MeOH, reflux, 8 h. ii. $NH_3{\cdot}H_2O$, 33% (two steps). (c) CS_2, K_3PO_4, DMF, R^2Br, room temperature (rt), overnight.

Scheme 3. Synthetic route to probe **10**. Reagents and conditions: (a) 4-cyanobenzylbromide, EtOH, reflux, 18 h, 85%. (b) **2**, CS_2, K_3PO_4, DMF, room temperature (rt), 4 h, 60%. (c) i. TFA, DCM, rt, 1 h; ii. $NH_3{\cdot}H_2O$, 80%. (d) D-(+)-biotin, EDCI, TEA, DMF, rt, 12 h, 20%.

Scheme 4. Synthetic route to compound **11** and the control probe (**14**). Reagents and conditions: (a) 4-Cyanobenzylamine, DMF, room temperature (rt), 24 h, 70%. (b) **7**, DMF, rt, 4 h, 82%. (c) i. TFA, DCM, rt, 1 h; ii. $NH_3{\cdot}H_2O$; 86%. (d) D-(+)-biotin, EDCI, TEA, DMF, rt, 24 h, 68%.

Fig. 2. The *E*- and *Z*-rotamers for intermediates **8**, **9** and probe **10**.

Fig. 3. Effects of compound **3n** on the cell-cycle distribution in A549 cells. (A) A549 cells were treated with 5-FU (IC_{50}), taxol (0.10 μM), or increasing concentrations of compound **3n** (0.5×, 1× or 2× IC_{50}) for 24 h before being harvested and analyzed by flow cytometry. Data are expressed as the means ± SD of three independent experiments. $^{**}P < 0.001$ (Student's t-test). (B) Representative cell-cycle profiles from three independent experiments.

Fig. 4. Compound **3n** does not induce DNA damage. (A) Compound **3n** failed to induce γ-H2AX foci formation. A549 cells were treated with camptothecin (CPT), compound **3n** or DMSO (diluent for CPT and **3n**) for 2 or 12 h. Cells were fixed and immuno-stained with a mouse monoclonal antibody against γ-H2AX, and DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI). (B) Compound **3n** failed to increase γ-H2AX and pS345 CHK1 protein levels. A549 cells were treated as described in (A), and total cell lysates were harvested and immunoblotted (IB) with the indicated antibodies.

Fig. 5. Compound **3n** increases the mitotic cell population. A549 cells were treated with compound **3n** at the concentration equivalent to its IC_{50}, for different times (2, 4, 8, and 16 h) and stained with anti-phospho-histone H3 (serine 10). Propidium iodide was used for DNA staining. Nocodazole (340 nM) was used as a control. (A) Representative images of A549 cells treated with compound **3n** from three independent experiments. (B) Data are expressed as the means ± SD of three independent experiments. (C) Compound **3n** induced BubR1, histone H3

Ser10 phosphorylation in A549 cells. After A549 cells were treated with compound **3n** for 6, 12, 18 and 24 h, total lysates were harvested for immunoblotting (IB) with the indicated antibodies.

Fig. 6. Lung cancer A549 cell lysate was incubated with biotinylated probe **10** (final probe concentration in the lysate was 0.15 mM) for 4 h at 4 °C, and then incubated with streptavidin-coated Dynabeads for 1 h at 4 °C. The mixture was loaded onto magnetic beads, and the beads were washed with washing buffer six times before denaturation and separation by SDS−PAGE. Visualization of the separated proteins with silver staining agent showed the specific binding proteins with probe **10**. Left lane and right lane were lysate incubated with biotin and control probe **14**, but there was no obvious protein band. Middle lane was cell lysate incubated with probe **10**, and contained numerous visible protein bands. The band marked with * is a non-specific binding protein.

Fig. 7. Tubulin specifically binds to probe **10**. (A) Probe **10** pulled down tubulin from A549 cell lysate. A549 cell lysate was incubated with biotin, probe **10** and **14** overnight at 4 °C. After incubation with Dynabeads for 1 h, the mixture was loaded on a magnet and washed six times. The sample was then denatured in SDS sample buffer and separated by SDS−PAGE, followed by immunoblotting using an α-tubulin antibody. (B) Probe **10** directly interacts with tubulin. Porcine neuronal tubulin was incubated with biotin, probe **10** and **14** overnight at 4 °C. Streptavidin pull-down and immunoblotting were performed as described in (A).

Fig. 8. Compound **3n** inhibits tubulin polymerization *in vitro*. *In vitro* tubulin polymerization assays were performed using purified porcine neuronal tubulin, according to the manufacturer′s instructions. I, II and III

denote the nucleation phase, the growth phase, and the steady-state equilibrium phase of tubulin polymerization *in vitro*, respectively. Tubulin was incubated at 37 °C in the presence of vehicle (DMSO), taxol (10 μM), nocodazole (10 μM), and **3n** (5, 10 and 20 μM). Absorbance at 340 nm was measured for 60 min and presented as the increased polymerized microtubule. The data represent one of three independent experiments, all with similar results.

Fig. 9. Competitive binding of probe **10** to tubulin with taxol, but not nocodazole, vinblastine or colchicine. (A) Tubulin was preincubated with nocodazole, taxol, vinblastine, or colchicine for 2 h at 4°C. (B) Tubulin was preincubated with taxol for 2 h at 4°C, and treated with increasing concentrations of the probe, then SDS−PAGE and immunoblotting was performed. (C−F) Probe **10** was preincubated with purified tubulin, and SDS-PAGE and immunoblotting was performed. The beads binding the probe-protein complex incubated with different concentration of taxol (C), nocodazole (D), vinblastine (E), or colchicine (F) for 2 h at 4°C. Images are representative of three independent experiments.

Table 2. Mitotic indexes of A549 cells treated with Nocodazole and compound **3n**.

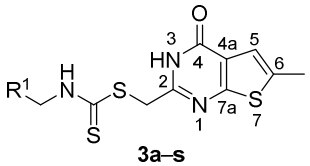
Compound	Mitotic index[a]			
	2 h	4 h	8 h	16 h
DMSO	2.67 ± 0.21	3.30 ± 0.17	3.17 ± 0.15	3.23 ± 0.15
Nocodazole	9.83 ± 0.06	14.57 ± 2.93	28.57 ± 8.06	57.93 ± 2.43
3n	6.63 ± 0.91	9.67 ± 3.91	15.60 ± 2.55	19.17 ± 2.97

[a] Data are expressed as mean ± SD from triplicate determination from three independent experiments. The mitotic index for untreated cells was 3.23 ± 0.06.

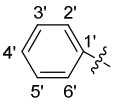
Table 3. Peptides were sequenced by Q Exactive HF LC/MS and internal sequences were searched pFind from the UniProt database using pFind.

Acc no.	Protein	Percent overage (%)	Thr. pI; *Mr* (Da)
F5H5D3	Tubulin α-1C chain	16.6	57,730.18
Q6PEY2	Tubulin α-3E chain	15.3	49,858.54
Q5JP53	Tubulin β chain	35.7	47,766.64
P68371	Tubulin β-4B chain	34.2	49,831.01
Q13509	Tubulin β-3 chain	16.7	50,432.68
Q92499	ATP-dependent RNA helicase DDX1	38.8	82,432.14
H3BLZ8	Probable ATP-dependent RNA helicase DDX17	52.8	80,439.64
Q9NR30	Nucleolar RNA helicase 2	35.1	87,344.40
Q14694-2	Isoform 2 of Ubiquitin carboxyl-terminal hydrolase 10	42.8	92,597
3KTL2	Serine/arginine-rich-splicing factor 1	52.2	28,329.18
Q13242	Serine/arginine-rich splicing factor 9	63.8	25,542.24
H7BY36	RNA-binding protein EWS (Fragment)	55.8	32,189.82
Q01844-6	Isoform 6 of RNA-binding protein EWS	40.5	62,507.83

Table 1. IC_{50} values for compounds **3a–s** in cultured A549 and HCT-116 cells.



3a–s

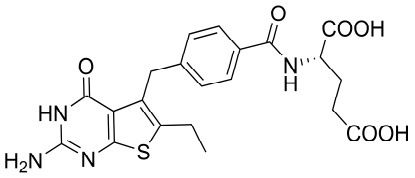


3a–o: R^1 =

3p–s: R^1 = heterocyclyl groups

Compound	R^1	IC_{50} (μM)[a]	
		A549	HCT-116
3a	C_6H_5	29.53 ± 0.57	> 30
3b	4'-$CH_3C_6H_4$	26.46 ± 0.95	28.00 ± 1.83
3c	4'-$OCH_3C_6H_4$	29.11 ± 1.03	24.54 ± 4.40
3d	2'-$OCH_3C_6H_4$	7.01 ± 0.12	18.05 ± 1.53
3e	2',4'-di$OCH_3C_6H_3$	> 30	> 30
3f	3',4',5'-tri$OCH_3C_6H_2$	7.89 ± 0.13	14.88 ± 2.02
3g	3',4'-MethylenedioxyC_6H_3	14.03 ± 0.45	24.61 ± 0.95
3h	4'-BrC_6H_4	21.90 ± 2.00	28.66 ± 1.25
3i	4'-ClC_6H_4	11.27 ± 0.74	25.12 ± 3.37
3j	2',4'-diClC_6H_3	29.51 ± 1.58	> 30
3k	4'-FC_6H_3	18.89 ± 0.56	28.67 ± 2.81
3l	2'-FC_6H_4	> 30	29.66 ± 1.36
3m	2',4'-diFC_6H_3	16.22 ± 0.57	> 30
3n	4'-CNC_6H_4	4.78 ± 0.12	22.59 ± 0.52
3o	4'-$NO_2C_6H_4$	12.30 ± 0.24	20.19 ± 2.14
3p	Thiophen-2-yl	22.96 ± 0.91	27.84 ± 1.70
3q	Furan-2-yl	> 30	29.84 ± 3.43
3r	Pyridin-3-yl	5.79 ± 0.49	9.30 ± 1.03
3s	Pyridin-4-yl	7.43 ± 0.27	14.10 ± 2.45
5-FU		3.52 ± 0.46	5.53 ± 0.90

[a] IC_{50}: The concentration that causes 50% inhibition of cell proliferation. Data are expressed as the means ± SD from triplicate determination from three independent experiments.



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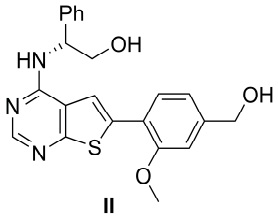
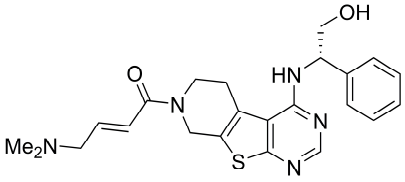


II



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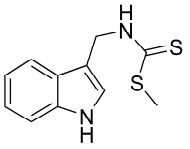
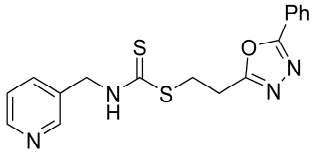
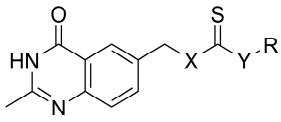


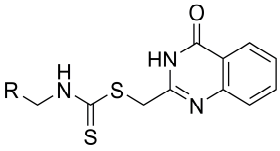
Brassinin



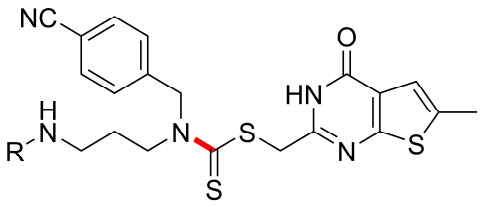
IV



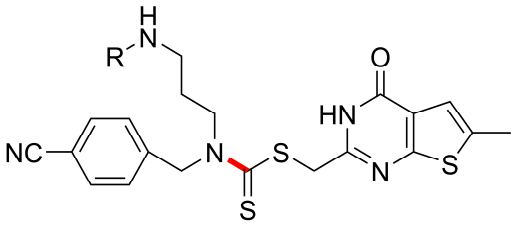
V, X = S, Y = NH
VI, X = NH, Y = S



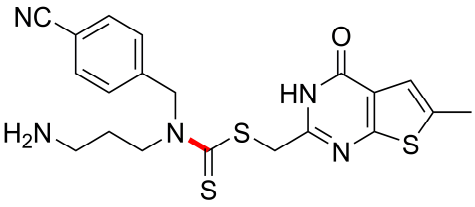
VII
R = 4-$CH_3OC_6H_4$



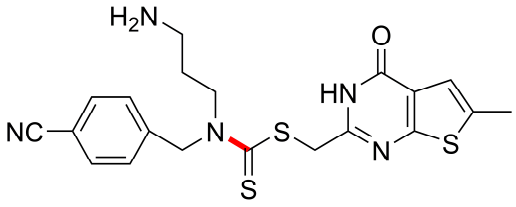
8, R = Boc
10, R = Biotinyl
E-rotamer, ca. 60%



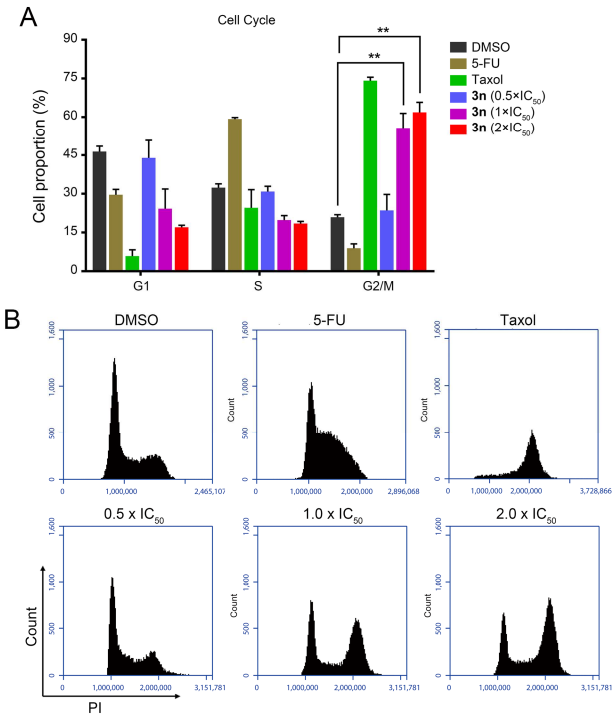
8, R = Boc
10, R = Biotinyl
Z-rotamer, ca. 40%

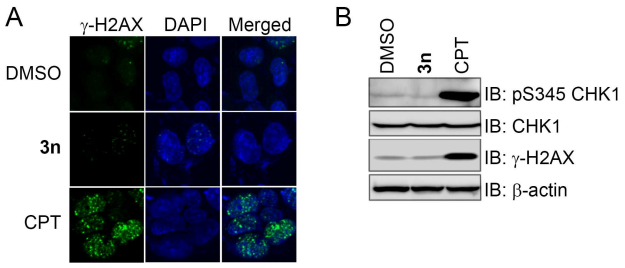


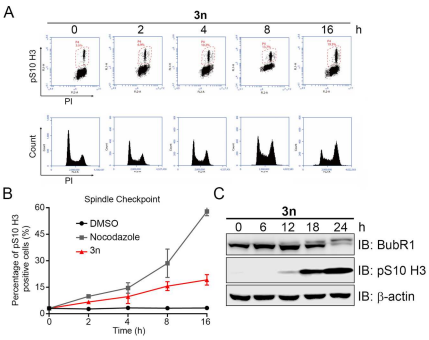
9
E-rotamer, ca. 50%

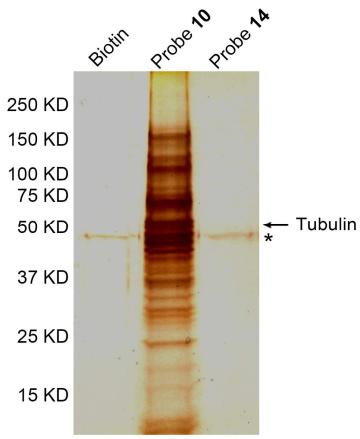


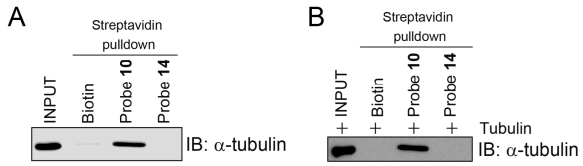
9
Z-rotamer, ca. 50%

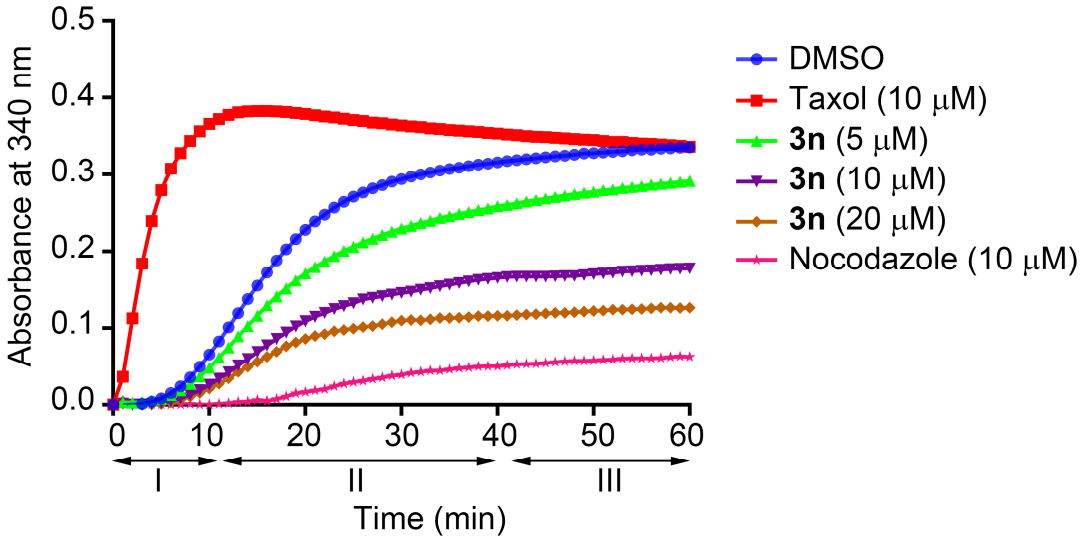


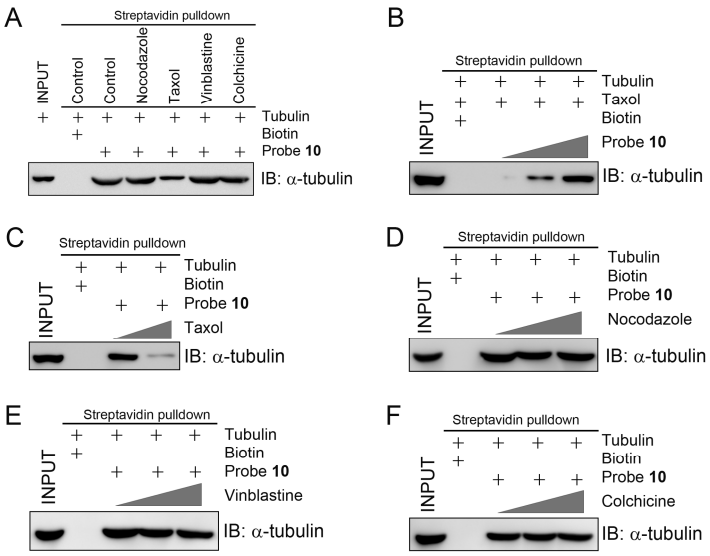


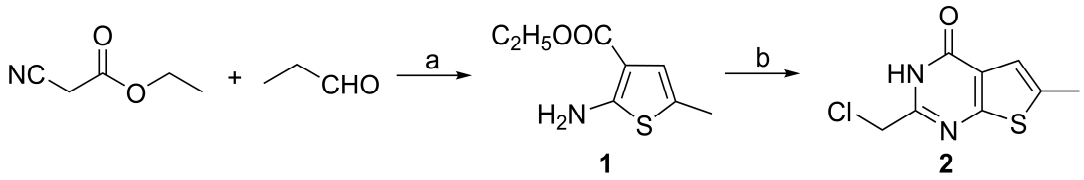


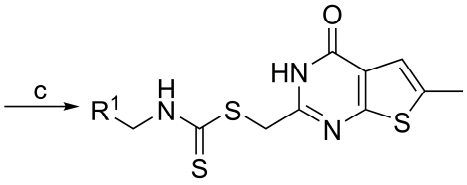




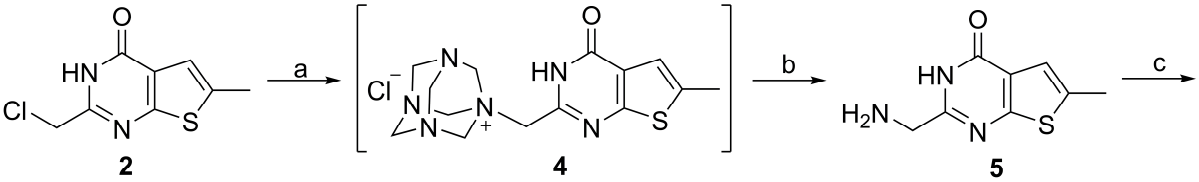


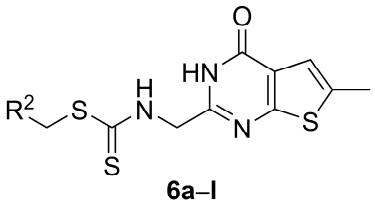




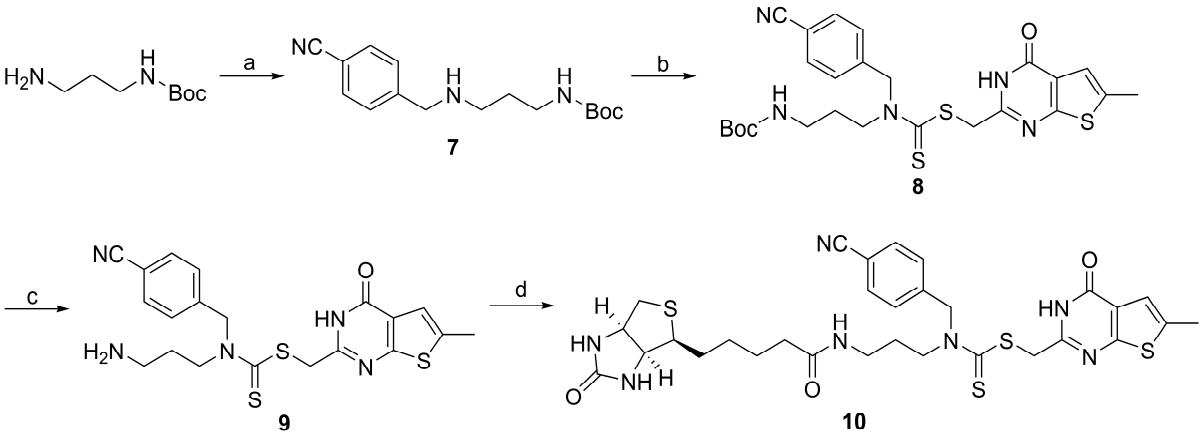


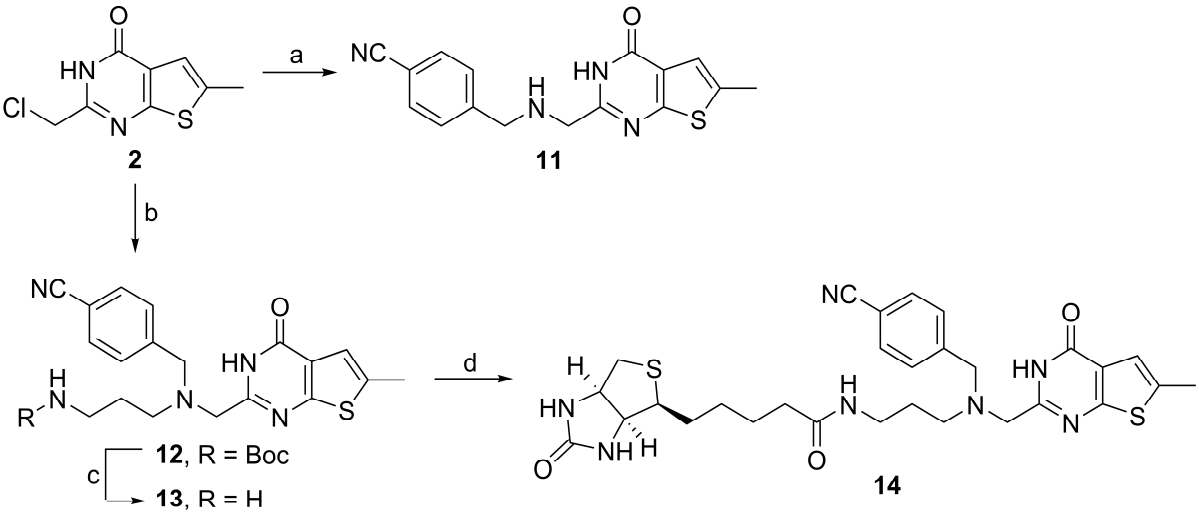
a. $R^1 = C_6H_5$
b. $R^1 = 4'\text{-}CH_3C_6H_4$
c. $R^1 = 4'\text{-}OCH_3C_6H_4$
d. $R^1 = 2'\text{-}OCH_3C_6H_4$
e. $R^1 = 2',4'\text{-}diOCH_3C_6H_3$
f. $R^1 = 3',4',5'\text{-}triOCH_3C_6H_2$
g. $R^1 = 3',4'\text{-}CH_2OCH_2C_6H_3$
h. $R^1 = 4'\text{-}BrC_6H_4$
i. $R^1 = 4'\text{-}ClC_6H_4$
j. $R^1 = 2',4'\text{-}diClC_6H_3$
k. $R^1 = 4'\text{-}FC_6H_3$
l. $R^1 = 2'\text{-}FC_6H_4$
m. $R^1 = 2',4'\text{-}diFC_6H_3$
n. $R^1 = 4'\text{-}CNC_6H_4$
o. $R^1 = 4'\text{-}NO_2C_6H_4$
p. R^1 = Thiophen-2-yl
q. R^1 = Furan-2-yl
r. R^1 = Pyridin-3-yl
s. R^1 = Pyridin-4-yl





a. $R^2 = C_6H_5$
b. $R^2 = 4'\text{-}CH_3C_6H_4$
c. $R^2 = 4'\text{-}OCH_3C_6H_4$
d. $R^2 = 3'\text{-}OCH_3C_6H_4$
e. $R^2 = 2'\text{-}OCH_3C_6H_4$
f. $R^2 = 4'\text{-}FC_6H_4$
g. $R^2 = 2',4'\text{-}diF_2C_6H_3$
h. $R^2 = 4'\text{-}ClC_6H_4$
i. $R^2 = 4'\text{-}BrC_6H_4$
j. $R^2 = 4'\text{-}NO_2C_6H_4$
k. $R^2 = 4'\text{-}CNC_6H_4$
l. $R^2 = 4'\text{-}EtO_2CC_6H_4$





Research highlights

- Two series of dithiocarbamate derivatives of thieno[2,3-*d*]pyrimidine were synthesized.
- **3n** was most cytotoxic against A549 cells, inducing G2/M phase arrest and activating the SAC.
- **3n** inhibited tubulin polymerization *in vitro* in a dose-dependent manner.
- Probe **10** (biotinylated **3n**) was synthesized to identify binding proteins by biotin-streptavidin affinity purification and MS analysis.
- Tubulin was the only identified protein related to the SAC that directly binds to probe **10**.
- **3n** competes with taxol in binding to tubulin, exerting cytotoxicity toward taxol-resistant cancer cells.