Substituted thiophene compounds as d-dopachrome tautomerase inhibitors

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(54) Title: SUBSTITUTED THIOPHENE COMPOUNDS AS D-DOPACHROME TAUTOMERASE INHIBITORS

(57) Abstract: The present invention relates to substituted thiophene compounds and methods useful for inhibiting D-dopachrome tautomerase. The invention also provides pharmaceutically acceptable compositions comprising compounds of the present invention and methods of using said compositions in the treatment of various disorders, notably cancer, such as NSCLC, and inflammation.

Substituted thiophene compounds as D-dopachrome tautomerase inhibitors

The present invention relates to substituted thiophene compounds and methods useful for inhibiting D-dopachrome tautomerase (DDT or MIF2). The invention also provides pharmaceutically acceptable compositions comprising compounds of the present invention and methods of using said compositions in the treatment of various disorders, notably cancer, such as NSCLC, and inflammation.

Background Art

Cancer is one of the major public health challenges, which contributes to an estimated annual death toll of ten million worldwide in recent years. Although targeted cancer treatment has achieved enormous progress over the last decades, its effectiveness is limited by the heterogeneity and acquired therapy resistance of cancers. Therefore, it is important to explore novel anti-cancer drug targets and to develop new therapeutic agents to target them. This could expand the possibilities to employ targeted therapeutic approaches and also increases the possibilities to develop combination therapy regimens.

The macrophage migration inhibitory factor (MIF) family proteins are implicated in the development of cancers, which is demonstrated by the overexpression of MIF family proteins in several cancer types, such as genitourinary cancer, melanoma, neuroblastoma, and lung carcinoma. Down-regulation of MIF family proteins by gene-knockout or gene-knockdown has not only reduced tumor progression and metastases, but also induced antitumor immune responses. Therefore, targeting the MIF family is a promising strategy towards development of novel cancer therapeutics.

The most studied member of the MIF family proteins is macrophage migration inhibitory factor (MIF, UniProtKB nr. P14174 (MIF_HUMAN)), which was initially discovered as an inflammatory cytokine. Through interfering with the interactions between MIF and its binding partners, several MIF-targeting reagents showed substantial potency on MIF-related signalling pathway deactivation and cancer cell proliferation inhibition. As a result, significant efforts have been invested into the development of MIF-targeting therapies. This afforded several MIF directed therapeutics that are now under pre-clinical and clinical investigation.

D-dopachrome tautomerase (DDT or MIF2, UniProtKB nr. P30046 (DOPD_HUMAN)) is a structural and functional homolog, but not a backup of MIF (see e.g. Sugimoto et al., *Biochemistry* **1999**, *38*, 3268–3279, and Illescas et al., *Cytokine* **2020**, *133*). MIF2 and MIF share a high similarity in several aspects. Firstly, the 3D structure shows that the overall folding and a subunit topology of MIF2 and MIF are almost identical, with two β-α-β motifs related by pseudo-2-fold symmetry and similar trimeric β-sheet packing. Secondly, both MIF2 and MIF harbour enzyme activity to catalyze the keto-enol tautomerization of 4-hydroxylphenylpyruvate (4-HPP) in an active site centered around 1-proline. Finally, both MIF and MIF2 are ligands of CD74 and JAB, that could consequently endow these two proteins with a similar effect on cell growth and tumorigenesis. However, despite their high structure similarity, MIF and MIF2 share just 34% percent amino acid sequence identity. These sequence differences provide differences in interaction sites. For instance, the difference of amino acids inside the tautomerase active sites cause differences in their activity towards keto-enol tautomerisation of 4-HPP. Moreover, MIF2 does not bind to MIF receptors CXCR2/4 because it lacks pseudo(E)LR motifs which mediate the interactions.

It has become clear that MIF2 plays a role that is similar or of even more importance than MIF in cell proliferation. Nevertheless, in contrast to MIF, the development of MIF2-directed therapeutics is lagging behind. The development of small-molecule inhibitors of MIF2 is almost non-explored. So far, 4-(3-carboxyphenyl)-2,5-pyridinedicarboxylic acid (4-CPPC) is the only reversible inhibitor reported to bind to MIF2. As reported in 2019, Bucala et al. (*J. Biol. Chem.* **2019**, *294*, 18522–18531) discovered selective MIF2 inhibitor 4-CPPC through virtual screening with an IC_{50} value of 27 μM on MIF2 tautomerase activity. More importantly, 4-CPPC can also inhibit MIF2-CD74 binding and MIF2 mediated activation of the MAPK pathway as determined by ERK phosphorylation.

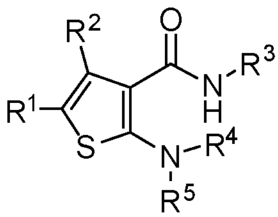
Apart from 4-CPPC as reversible MIF2 inhibitor The only covalent inhibitor reported to bind to MIF2 is 4-iodo-6-phenylpyrimidine (4-IPP) (Rajasekaran et al., *FASEB J.* **2014**, *28*, 4961–4971). 4-IPP covalently binds to Pro1 of MIF2 to interfere with its tautomerase enzyme activity and its biological function. However, 4-IPP shows low potency on MIF2 inhibition with an IC_{50} value larger than 100 μM. In contrast, 4-IPP inhibits MIF with micromolar potency and binds covalently to the active site proline.

An over-activation of the MAP/ERK and/or the PI3K/Akt pathway by MIF family member proteins leads to the uncontrolled growth of many cancers (*Med Res Rev.* 2016, 36(3), 440-640), including melanoma, acute myeloid leukemia (AML), non-small cell lung cancer (NSCLC), colorectal cancer, ovarian cancer, thyroid cancer, hairy cell leukemia, prostate cancer, glioblastoma, breast cancer, and oral cancer (*Int. J. Mol. Sci.* 2020, 21, 1102; *BMC Cancer*, 2014, 14(1), 30). Targeting the MAPK pathway in these cancers has provided enormous success in cancer treatment. MIF2 is one of the key activators of the MAP/ERK pathway (*PNAS* 2011, 108, E577) and was implicated in the pathogenesis of several types of cancers, such as neuroblastoma (*Brain Sci.* 2019, 9, 284), melanoma (*FASEB J.* 2021, 35, e21671), renal tumor (*JBC* 2014, 289, 3713), pancreatic cancer (*Int. J. Cancer* 2016, 139, 2056), NSCLC (*J. Immunol.* 2008, 181, 2330), glioblastoma (*Oncol Lett.* 2018, 16, 2881), and genitourinary cancer (*Nat. Rev. Urol.* 2019, 16, 318). Therefore, a promising strategy for cancer treatment is via tackling MIF2 activated MAPK pathway over-activation.

To further investigate the biological role of MIF2 in inflammation and cancer and to exploit it to develop therapy for inflammatory diseases and cancer, more potent MIF2 inhibitors are needed. It is an objective of the present invention to provide a MIF2 inhibitor, preferably a MIF2 inhibitor with an IC_{50} value of 100 µM or less, even more preferably less than 27 µM, most preferably less than 8 µM. It is a further objective of the present invention to provide a selective MIF2 inhibitor.

Description of the invention

The present invention relates to compounds of structure (I)



(I)

or pharmaceutically acceptable salts thereof, wherein

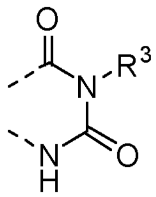
R^1 represents H or optionally substituted phenyl;

R^2 represents H, straight or branched C_{1-8} alkyl, straight or branched C_{2-8} alkenyl, or optionally substituted phenyl;

R^3 represents straight or branched C_{1-8} alkyl, straight or branched C_{2-8} alkenyl, phenyl, benzyl, phenethyl, naphthyl, naphthalen-1-ylmethyl, naphthalen-2-ylmethyl, naphthalen-1-yl-2-ethyl, or naphthalen-2-yl-2-ethyl, all optionally substituted with one or more Cl, F, Br, CH_3, C_2H_5, CCl_3, CF_3 or CBr_3 groups;

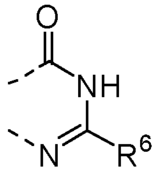
R^5 represents H, and R^4 represents H or C_{1-8} alkanoyl; or

R^3, the amide group to which R^3 is attached, R^4, R^5, and the nitrogen atom to which R^4 and R^5 are attached together form a bivalent radical with structure



wherein R^3 is as defined above; or

R^3, the amide group to which R^3 is attached, R^4, R^5, and the nitrogen atom to which R^4 and R^5 are attached together form a bivalent radical with structure



wherein R^6 represents H, Cl, F, Br, or

wherein R^6 represents straight or branched C_{1-8} alkyl, straight or branched C_{2-8} alkenyl, phenyl, benzyl, phenethyl, naphthyl, naphthalen-1-ylmethyl, naphthalen-2-ylmethyl, naphthalen-1-yl-2-ethyl, or naphthalen-2-yl-2-ethyl, all optionally substituted with one or more Cl, F, Br, C_{1-4} alkyl, C_{1-4} alkoxy, CCl_3, CF_3 or CBr_3 groups.

The invention further relates to a pharmaceutical composition comprising a compound or a pharmaceutically acceptable salt thereof according to the invention, and a pharmaceutically acceptable adjuvant, carrier or vehicle.

The term “pharmaceutically acceptable adjuvant, carrier or vehicle” refers to a nontoxic carrier, adjuvant or vehicle that does not destroy the pharmacological activity of the compound with which it is formulated. Pharmaceutically acceptable carriers, adjuvants or vehicles that are used in the compositions of this invention include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat.

The invention further relates to a compound or pharmaceutically acceptable salt thereof or a pharmaceutical composition according to the invention for use as a medicament.

The invention further relates to a compound or pharmaceutically acceptable salt thereof or a pharmaceutical composition according to the invention for use in the treatment of a medical condition.

The invention further relates to a compound or pharmaceutically acceptable salt thereof or a pharmaceutical composition according to the invention for use in therapy.

The invention further relates to a compound or pharmaceutically acceptable salt thereof or a pharmaceutical composition according to the invention for use as medicament, wherein the use is as a MIF2 inhibitor.

The invention further relates to a compound or pharmaceutically acceptable salt thereof or a pharmaceutical composition according to the invention for use in treating cancer or inflammatory disease.

Inflammatory diseases include a vast array of disorders and conditions that are characterized by inflammation. Examples include allergy, asthma, autoimmune diseases, coeliac disease, glomerulonephritis, hepatitis, inflammatory bowel disease, preperfusion injury and transplant rejection.

Cancers that may particularly well be treated with the compounds of the invention include melanoma, AML, NSCLC, colorectal cancer, ovarian cancer, thyroid cancer, hairy cell leukemia, prostate cancer, glioblastoma, breast cancer, and oral cancer, neuroblastoma, renal tumors, pancreatic cancer and genitourinary cancer. AML and/or NSCLC may particularly well be treated with the compounds of the invention.

The invention further relates to a compound or pharmaceutically acceptable salt thereof or a pharmaceutical composition according to the invention for use in treating AML or NSCLC, most preferably for use in treating NSCLC.

The invention further relates to a compound or pharmaceutically acceptable salt thereof or a pharmaceutical composition according to the invention for use in treating a medical disorder, preferably a disorder in which MIF2 inhibition relieves the pathological process, more preferably a disorder in which activation of the MAP/ERK and/or the PI3K/Akt pathway results in uncontrolled cell proliferation. Such a disorder may in certain cases be characterized by overexpression of MIF2.

Disorders in which MIF2 inhibition relieves the pathological process, are generally disorders in which inhibition of cell proliferation relieves the pathological process. This is for example the case in cancers and inflammatory diseases.

The invention further relates to a method for treating cancer or inflammation in a patient in need thereof, comprising administering to said patient an effective amount of a compound or pharmaceutically acceptable salt thereof or a pharmaceutical composition according to the invention.

The invention further relates to a method for treating NSCLC in a patient in need thereof, comprising administering to said patient an effective amount of a compound or pharmaceutically acceptable salt thereof or the pharmaceutical composition according to the invention.

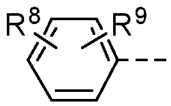
The invention further relates to a method for treating AML in a patient in need thereof, comprising administering to said patient an effective amount of a compound or pharmaceutically acceptable salt thereof or the pharmaceutical composition according to the invention.

The invention further relates to a method for treating a disorder characterized by overexpression of MIF2 in a patient in need thereof, comprising administering to said patient an effective amount of the compound or pharmaceutically acceptable salt thereof or the pharmaceutical composition according to the invention.

Compositions of the present invention may be administered orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally, vaginally or via an implanted reservoir. The term “parenteral” as used herein includes subcutaneous, intravenous, intramuscular, intra-articular, intra-synovial, intrasternal, intrathecal, intrahepatic, intralesional and intracranial injection or infusion techniques.

Description of embodiments

Preferably, R^1 represents



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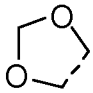
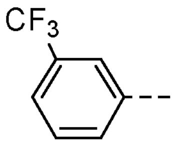
wherein R^8 and R^9 each independently represent H, F, Cl, Br, straight or branched C_{1-4} alkyl, CCl_3, CBr_3, or CF_3, or wherein R^8 and R^9 are attached to adjacent carbon atoms and together

with these adjacent carbon atoms form a five- or six-membered homo- or heterocyclic ring, for example a dioxolane ring with structure  .

In some aspects, at least one of R^8 and R^9 is F, Cl, Br, CCl_3, CBr_3, or CF_3. Preferably both of R^8 and R^9 are F, Cl, Br, CCl_3, CBr_3, or CF_3.

In some aspects, both R^8 and R^9 are F, Cl or Br, preferably both R^8 and R^9 are Cl.

In some aspects, one of R^8 and R^9 is H, and the other is CCl_3, CBr_3, or CF_3, preferably CF_3. It is especially preferred that R^1 represents



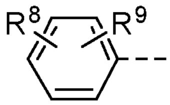
.

Preferably, R^2 represents H, straight or branched C_{1-4} alkyl or straight or branched C_{2-4} alkenyl. More preferably R^2 represents straight or branched C_{1-4} alkyl or straight or branched C_{2-4} alkenyl. Even more preferably R^2 represents methyl or ethyl.
It is especially preferred that R^2 represents methyl.

Preferably, when R^2 is unsubstituted phenyl, then R^3 is not unsubstituted phenyl, and vice versa.

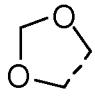
Preferably, R^3 represents benzyl, phenethyl, naphthyl, naphthalen-1-ylmethyl, naphthalen-2-ylmethyl, naphthalen-1-yl-2-ethyl, or naphthalen-2-yl-2-ethyl, all optionally substituted with one or more Cl, F, Br, CH_3, C_2H_5, CCl_3, CF_3 or CBr_3 groups.

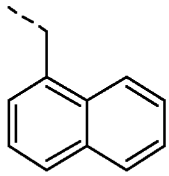
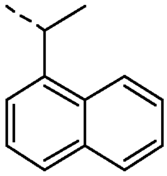
In a particularly preferred embodiment, R^1 represents

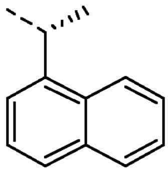


,

wherein R^8 and R^9 each independently represent H, F, Cl, Br, straight or branched C_{1-4} alkyl, CCl_3, CBr_3, or CF_3, or wherein R^8 and R^9 are attached to adjacent carbon atoms and together

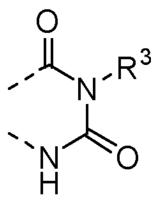
with these adjacent carbon atoms form a five- or six-membered homo- or heterocyclic ring, for example a dioxolane ring with structure , and R^2 represents straight or branched C_{1-4} alkyl or straight or branched C_{2-4} alkenyl, most preferably methyl, and R^3 represents benzyl, phenethyl, naphthyl, naphthalen-1-ylmethyl, naphthalen-2-ylmethyl, naphthalen-1-yl-2-ethyl, or naphthalen-2-yl-2-ethyl, all optionally substituted with one or more Cl, F, Br, CH_3, C_2H_5, CCl_3, CF_3 or CBr_3 groups.

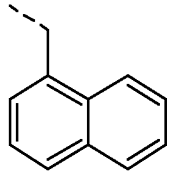
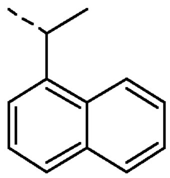
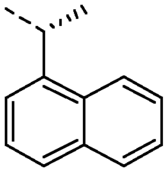
In some aspects, R^3 represents naphthalen-1-ylmethyl  or naphthalen-1-yl-2-ethyl .

It is especially preferred that R^3 represents .

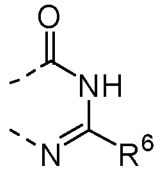
In some aspects, R^4 and R^5 represent H.

In some aspects, R^3, the amide group to which R^3 is attached, R^4, R^5, and the nitrogen atom to which R^4 and R^5 are attached together form a bivalent radical with structure



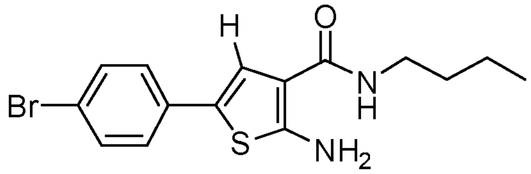
wherein R^3 represents naphthalen-1-ylmethyl  or naphthalen-1-yl-2-ethyl , preferably .

In some aspects, R^3, the amide group to which R^3 is attached, R^4, R^5, and the nitrogen atom to which R^4 and R^5 are attached together form a bivalent radical with structure

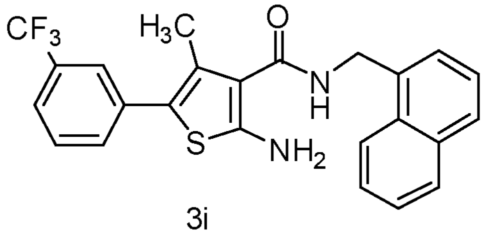


wherein R^6 represents Cl, F, Br, or phenyl optionally substituted with one or more Cl, F, Br, C_{1-4} alkyl, C_{1-4} alkoxy,, CCl_3, CF_3 or CBr_3 groups, preferably wherein R^6 represents phenyl substituted with one or more Cl, F, Br, C_{1-4} alkyl, C_{1-4} alkoxy, CCl_3, CF_3 or CBr_3 groups, more preferably wherein R^6 represents phenyl substituted with 3-OCH_3.

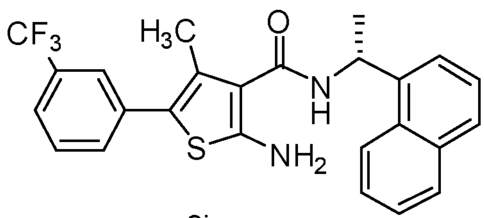
Highly preferred embodiments relate to the following compounds or pharmaceutically acceptable salts thereof:



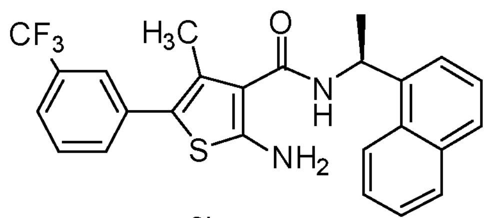
3b



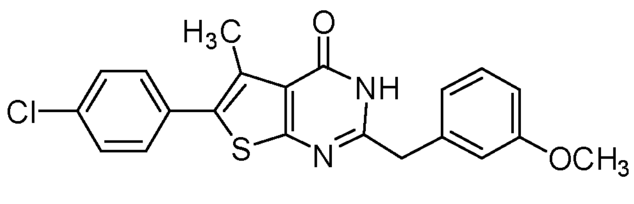
3i



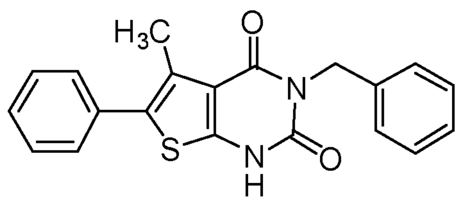
3j



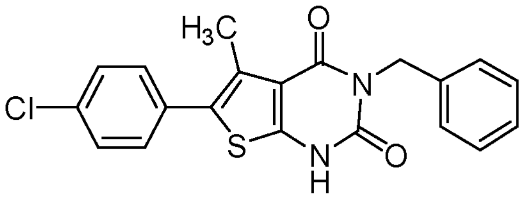
3k



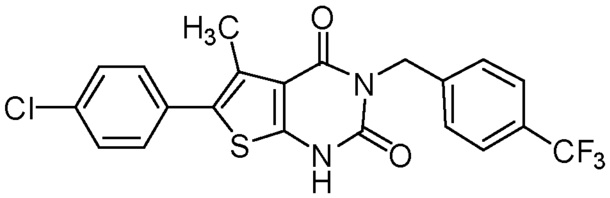
8d



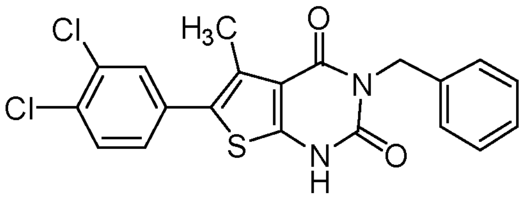
7b



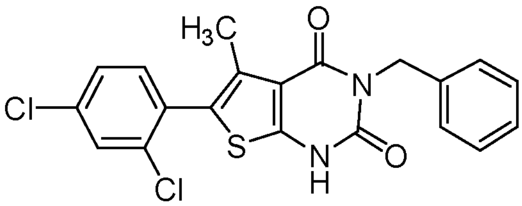
7d



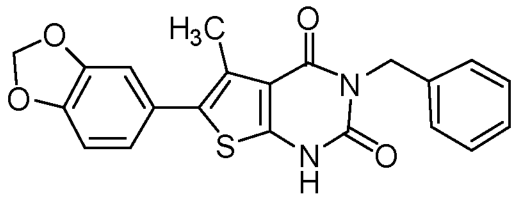
7e



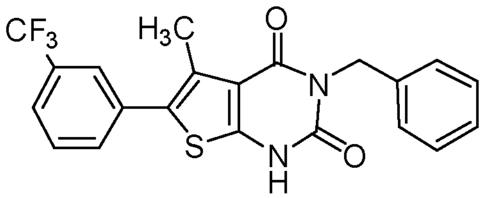
7f



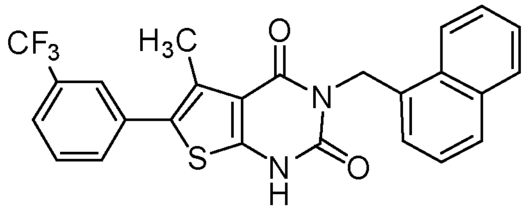
7g



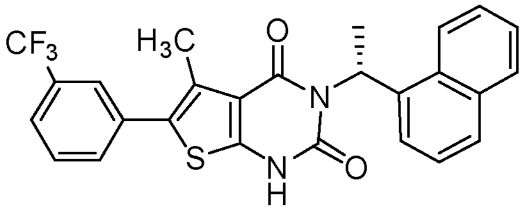
7i



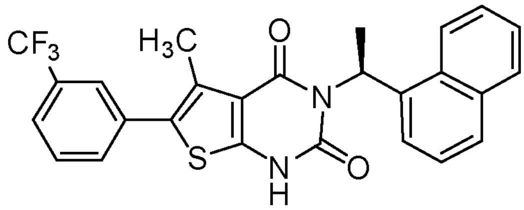
7h



5c



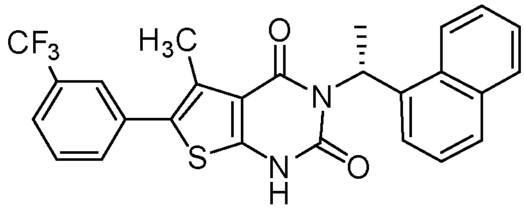
5d



5e

.

Particularly preferred embodiments relate to



5d

((R)-5-Methyl-3-(1-(naphthalen-1-yl)ethyl)-6-(3-(trifluoromethyl)phenyl)thieno[2,3-d]pyrimidine-2,4(1H,3H)-dione), or a pharmaceutically acceptable salt thereof.

In embodiments of the invention, the pharmaceutical compositions further comprises a MIF inhibitor. This enables dual targeting of MIF and MIF2 at the same time, which will provide a synergistic effect and thereby highly effective treatment of diseases in which inhibition of cell proliferation provides relief, such as cancer and inflammatory diseases.

Brief description of the figures

Figure 1 A-D display the results of NSCLC cell proliferation assays with **5d**.

Figure 2 A and B display the results of colony formation experiments with NSCLC cells treated with **5d**.

Figure 3 A and B display the results of growth experiments with A549 cancer cells treated with **5d** in a spheroid model.

Figure 4 A and B display the results of cell cycle arrest experiments with A549 cancer cells treated with **5d.**

Figure 5 A and B display the results of MIF2-induced ERK phosphorylation experiments in A549 cancer cells treated with **5d.**

Detailed description of the figures

Figure 1. **5d** treatment inhibits cell proliferation of NSCLC cells. (A) A549, (B) H1650, (C) H1299, (D) HCC827 cells were seeded with a density of 1000 cells per well in a 96-well plate. After overnight culturing, the cells were treated with various concentrations of **5d** for 72 hours. The resulting cells were quantified by CyQUANT® cell proliferation assays and compared with the vehicle (DMSO) control (n=3).

Figure 2. **5d** treatment inhibits colony formation of NSCLC cells. (A) A549 or other NSCLC cells were seeded in 12-well plate at a density of 1000 cells/well and incubated overnight. The cells were then treated with compounds or vehicle (DMSO) for 5 days. Afterwards, cells were fixed and stained with 0.5% crystal violet solution. The image of the representative well was scanned and shown. (B) The stained colonies were dissolved in 30% acetic acid and then quantified by measuring the absorbance at 590 nm. The relative colony number is normalized to the DMSO-treated control. The data shown are the average of triplicate samples with SD. (n = 3, *p<0.05, **p<0.01 and ***p<0.001 *vs* control).

Figure 3. **5d** treatment inhibits growth of A549 cancer cells in a spheroid model. A549 cells were seeded in an ultralow attachment 96-well round-bottomed plate (1000 cells/well) to generate tumor spheroids (a single spheroid per well). After initiation, the spheroids were treated with **5d** at the indicated concentrations every three days. DMSO was used as vehicle control. The day of the first treatment was indicated as day 0. (A) Representative images

were obtained at the indicated intervals using an inverted microscope. Scale bar: 500 µm. (B) Analysis was carried out using NIS-Elements AR 3.1 software, and growth curves were obtained relative to the untreated spheroids (day 0) and plotted with GraphPad Prism 8. Values are shown as means ± SD (n = 3 spheroids/time point, *p<0.05, **p<0.01 and ***p<0.001 *vs* control).

Figure 4. **5d** induces cell cycle arrest in A549 cells. (A) A549 cells were treated with **5d** at the indicated concentrations for 48 h. The graphs show the representative cell cycle distribution of propidium-iodide stained cells assessed by flow cytometry. (B) Relative number of cells in each stage of the cell cycle (G_0/G_1, S and G_2/M phases) were analyzed by FlowJo. Data are shown as mean±SD of three replicates. *t*-test analysis was performed between G2/M phase of treated groups and control group. *p<0.05 and **p<0.01 *vs* vehicle group.

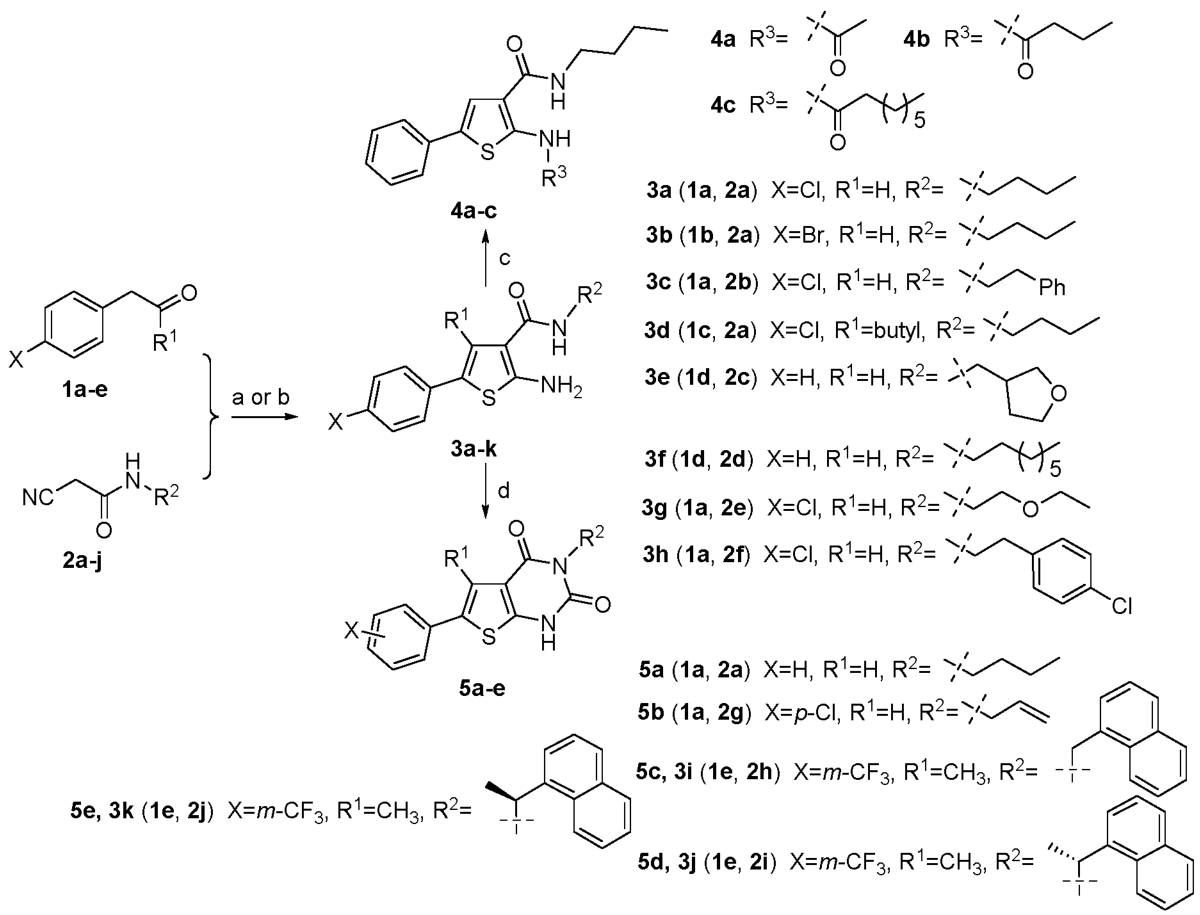
Figure 5. 5d inhibits the MIF2-induced ERK phosphorylation in A549 cells. (A) A549 cells were treated with MIF2 or **5d** pre-incubated MIF2 at indicated concentrations for 15 minutes, the pERK, total ERK and GAPDH was examined by immunoblots with anti-pERK, anti-ERK or anti-GAPDH antibodies. A representative western blot is shown. (n=2) (B) Quantification of the pERK level using pERK:ERK ratio, normalized to control group. GAPDH was used as a loading control on western blots. Data are shown as mean±SD. **p<0.01 and ***p<0.001 *vs* vehicle group.

EXPERIMENTAL SECTION

Synthesis routes

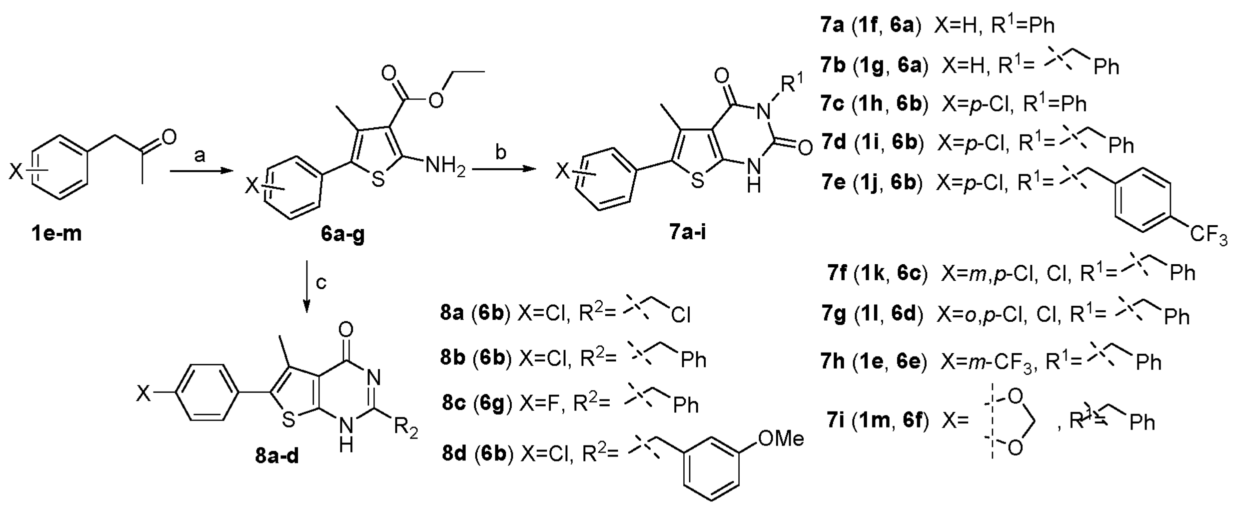
Several concise synthetic routes based on the highly reliable Gewald three-component reaction to construct phenylthiophene derivatives were utilized. The experimental details are depicted in Schemes 1−3. Compounds **3a-c**, **3e-h** were obtained from a one-pot reaction using different aldehydes, cyanoacetamides and elementary sulfur as starting materials with yields of 18-56%. To synthesize **3d**, **3i-k**, ketones were firstly reacted with cyanoacetamides in the presence of $SnCl_4$ and Et_3N to form the corresponding intermediates, which were cyclized with S_8 to afford the products with overall yields of 16-42%. The 2-aminothiophenes were acylated by different acylchlorides to prepare the desired 2-amide substituted products **4a-c** with yields of 44-58%. The 2-aminothiophenes were also employed to synthesize thieno[2,3-*d*]pyrimidine-2,4(1*H*,3*H*)-diones **5a-e** using 1,1'-carbonyldiimidazole (CDI) as coupling reagent with yields of 40-91%. Same scaffold in **7a-i** and **11a-b** was synthesized using different method, in which isocyanates were reacted with 2-aminothiophenes to make ureas before cyclized by MeONa with overall yields of 20-62%. **8a-d** were constructed by the condensation of 2-aminothiophenes with nitriles using 4N HCl with yields of 32-60%. All final compounds were purified with chromatography and characterized by ^{1}H and ^{13}C NMR spectroscopy and LC-HRMS.

Scheme 1. Synthesis of thiophene derivatives.[a]



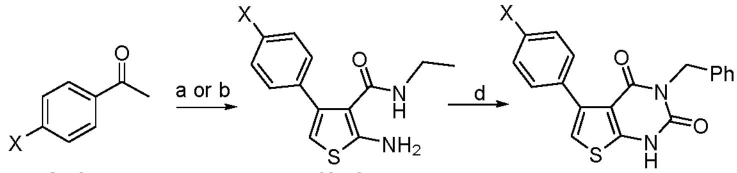
[a]Reagents and conditions: a. S_8, TEA, EtOH, reflux; b. i) acetic acid, toluene, reflux; ii) S_8, EtOH, reflux; c. Acyl chloride, pyridine, DMF, rt; d. CDI, CH_2Cl_2.

Scheme 2. Synthesis of thiophene derivatives.[a]



[a]Reagents and conditions: a. S_8, TEA, EtOH, reflux; b. i) R-NCO, pyridinee, reflux; ii) 30% MeONa, reflux; c. R-CN, HCl, dioxane, reflux.

Scheme 3. Synthesis of thiophene derivatives.[a]



9a-b **10a-b** **11a** X=H **11b** X=Cl

[a]Reagents and conditions: a. S_8, TEA, EtOH, reflux; b. i) acetic acid, toluene, reflux; ii) S_8, EtOH, reflux; d. CDI, CH_2Cl_2.

General. All the reagents and solvents were purchased from Sigma-Aldrich, AK Scientific, Fluorochem or Acros and were used without further purification unless stated otherwise. Reactions were monitored by thin layer chromatography (TLC) by the use of Merck silica gel 60 F_{254} plates. Spots were visualized with UV light. MP Ecochrom silica 32-63, 60 Å was used for flash column chromatography. Nuclear magnetic resonance spectra, ^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz), were recorded on a Bruker Avance 500 spectrometer. ^{1}H NMR spectra were reported in parts per million (ppm) referenced to $CDCl_3$: δ = 7.26 ppm (^{1}H) and 77.05 ppm (^{13}C) or DMSO-d6: δ = 2.50 ppm (^{1}H) and 39.52 ppm (^{13}C). The following abbreviations were used for spin multiplicity: s (singlet), d (doublet), t (triplet), q (quartet), dd (double of doublets), and m (multiplet). Coupling constants were reported in Hertz (Hz). High-resolution mass spectra were recorded using Fourier Transform Mass Spectrometry (FTMS) and electrospray ionization (ESI) on an Applied Biosystems/SCIEX API3000-triple quadrupole mass spectrometer. Purity of the compounds was determined by C18 reverse-phase high-performance liquid chromatography (HPLC) analysis to be >95%.

2-Amino-N-butyl-5-(4-chlorophenyl)thiophene-3-carboxamide, 3a. The starting materials **1a-e** and **2a-j** were either purchased or prepared using previous published methods (Eleftheriadis et al., *Eur. J. Med. Chem.* **2016**, *122*, 786–801).To synthesize **3a**, 2-(4-chlorophenyl)acetaldehyde (**1a**, 0.3 g, 2.0 mmol) in ethanol (5 mL) was added into a stirred solution of N-butyl-2-cyanoacetamide (**2a**, 0.3 g, 2.1 mmol). To the resulting suspension, triethylamine (0.3 mL, 2.1 mmol) and S_8 (80mg, 0.3 mmol) were added and the reaction mixture was refluxed overnight. After cooled down to room temperature, the mixture was diluted with EtOAc (50 mL) and washed with water (2 × 30 mL) and brine (2 × 30 mL). The organic layer was collected and dried over $MgSO_4$, filtrated and the solvent was removed under reduced pressure. Further purification by flash chromatography, with petroleum ether/EtOAc 5:1 (v/v) as eluent. 260 mg product was obtained as light-brown solid, yield 42%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.70 (t, J = 5.6 Hz, 1H), 7.64 (s, 1H), 7.50 (s, 2H), 7.43 – 7.35 (m, 4H), 3.18 (q, J = 6.9 Hz, 2H), 1.47 (p, J = 7.3 Hz, 2H), 1.35 – 1.29 (m, 2H), 0.90 (t, J

= 7.3 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 165.15, 161.12, 133.28, 129.92, 128.94, 125.09, 121.76, 119.86, 108.33, 38.04, 31.58, 19.71, 13.80. HRMS, calculated for $C_{15}H_{18}ON_2ClS$ [M + H]$^+$: 309.0823, found 309.0824.

2-Amino-5-(4-bromophenyl)-N-butylthiophene-3-carboxamide, 3b. 2-(4-bromophenyl)acetaldehyde (0.2 g, 1.0 mmol) and N-butyl-2-cyanoacetamide (140 mg, 1.0 mmol) were reacted using a procedure similar to the synthesis of 3a, affording compound 3b (97 mg, yield 28%) as a light-yellowish powder. ^1H NMR (500 MHz, DMSO-d_6) δ 7.71 (t, J = 5.6 Hz, 1H), 7.66 (s, 1H), 7.53 (d, J = 8.6 Hz, 2H), 7.32 (d, J = 8.6 Hz, 2H), 3.18 (q, J = 6.9 Hz, 2H), 1.47 (m, 2H), 1.31 (m, 2H), 0.90 (t, J = 7.3 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 165.6, 161.6, 134.1, 132.3, 125.8, 122.3, 120.3, 118.7, 108.8, 38.5, 32.0, 20.2, 14.2. HRMS, calculated for $C_{15}H_{18}ON_2BrS$ [M + H]$^+$: 353,0318, found 353,0318.

2-Amino-5-(4-chlorophenyl)-N-phenethylthiophene-3-carboxamide, 3c. 2-(4-chlorophenyl)acetaldehyde (**1a**, 77 mg, 0.5 mmol) and 2-cyano-N-phenethylacetamide (**2b**, 94 mg, 0.5 mmol) were reacted using a procedure similar to the synthesis of 3a, affording compound 3c (33 mg, yield 18%) as a light-yellowish powder. ^1H NMR (500 MHz, DMSO-d_6) δ 7.88 (t, J = 5.4 Hz, 1H), 7.62 (s, 1H), 7.52 (s, 2H), 7.41 (d, J = 8.6 Hz, 2H), 7.37 (d, J = 8.7 Hz, 2H), 7.30 (t, J = 7.3 Hz, 2H), 7.22 (dd, J = 16.7, 7.0 Hz, 3H), 3.43 – 3.38 (m, 2H), 2.81 (t, J = 7.5 Hz, 2H). ^{13}C NMR (126 MHz, DMSO) δ 165.20, 161.29, 139.65, 133.24, 129.95, 129.00, 128.93, 128.64, 128.39, 126.09, 125.06, 119.90, 108.21, 40.23, 35.55. MS (ESI): m/z 357.15 [M+H]$^+$.

2-Amino-5-phenyl-N-((tetrahydrofuran-3-yl)methyl)thiophene-3-carboxamide, 3e. 2-phenylacetaldehyde (**1d**, 60 mg, 0.5 mmol) and 2-cyano-N-((tetrahydrofuran-3-yl)methyl)acetamide (**2c**, 84 mg, 0.5 mmol) were reacted using a procedure similar to the synthesis of 3a, affording compound **3e** (52 mg, yield 35%) as a light-yellowish powder. ^1H NMR (500 MHz, DMSO-d_6) δ 7.82 (t, J = 5.8 Hz, 1H), 7.67 (s, 1H), 7.46 (s, 2H), 7.40 (d, J = 8.0 Hz, 2H), 7.34 (t, J = 7.8 Hz, 2H), 7.16 (t, J = 7.3 Hz, 1H), 3.93 (p, J = 6.3 Hz, 1H), 3.77 (q, J = 7.1, 6.2 Hz, 1H), 3.62 (q, J = 7.4 Hz, 1H), 3.25 (t, J = 5.9 Hz, 2H), 1.91 – 1.77 (m, 3H), 1.62 – 1.52 (m, 1H). ^{13}C NMR (126 MHz, DMSO) δ 165.38, 161.03, 134.30, 128.98, 125.84, 123.58, 121.37, 120.97, 108.01, 77.35, 67.20, 42.59, 28.66, 25.15. HRMS, calculated for $C_{16}H_{19}O_2N_2S$ [M + H]$^+$: 303.1162, found 303.1162.

2-Amino-N-octyl-5-phenylthiophene-3-carboxamide, 3f. 2-phenylacetaldehyde (**1d**, 60 mg, 0.5 mmol) and 2-cyano-N-octylacetamide (**2d**, 0.1 g, 0.5 mmol) were reacted using a procedure similar to the synthesis of 3a, affording compound **3f** (72 mg, yield 44%) as a light-yellowish powder. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.71 (t, J = 5.6 Hz, 1H), 7.60 (s, 1H), 7.43 (s, 2H), 7.39 (d, J = 7.3 Hz, 2H), 7.34 (t, J = 7.7 Hz, 2H), 7.16 (t, J = 7.7 Hz, 1H), 3.17 (q, J = 6.7 Hz, 2H), 1.52 – 1.43 (m, 2H), 1.30 – 1.23 (m, 10H), 0.85 (t, J = 6.8 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 165.23, 160.77, 134.30, 128.98, 125.83, 123.63, 121.35, 120.67, 108.25, 38.40, 31.32, 29.47, 28.86, 28.74, 26.60, 22.15, 14.00. HRMS, calculated for $C_{19}H_{27}N_2S$ [M + H]$^+$: 331.1839, found 331.1839.

2-Amino-5-(4-chlorophenyl)-N-(2-ethoxyethyl)thiophene-3-carboxamide, 3g. 2-(4-chlorophenyl)acetaldehyde (**1a**, 77 mg, 0.5 mmol) and 2-cyano-N-(2-ethoxyethyl)acetamide (**2e**, 80 mg, 0.5 mmol) were reacted using a procedure similar to the synthesis of 3a, affording compound 3g (88 mg, yield 56%) as a light-yellowish powder. ^{1}H NMR (500 MHz, Chloroform-d) δ 7.34 (d, J = 8.6 Hz, 2H), 7.29 (d, J = 8.6 Hz, 2H), 6.94 (s, 1H), 6.23 (s, 2H), 6.11 (s, 1H), 3.58 (d, J = 2.5 Hz, 4H), 3.55 (q, J = 7.0 Hz, 2H), 1.24 (t, J = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.78, 160.62, 132.68, 132.35, 129.12, 125.94, 123.98, 118.84, 109.92, 69.45, 66.65, 39.20, 15.32. HRMS, calculated for $C_{15}H_{18}ON_2$ ClS [M + H]$^+$: 325.0772, found 325.0773.

2-Amino-N-(4-chlorophenethyl)-5-(4-chlorophenyl)thiophene-3-carboxamide, 3h. 2-(4-chlorophenyl)acetaldehyde (**1a**, 77 mg, 0.5 mmol) and N-(4-chlorophenethyl)-2-cyanoacetamide (**2f**, 0.1 g, 0.5 mmol) were reacted using a procedure similar to the synthesis of 3a, affording compound 3g (66 mg, yield 34%) as a light-yellowish powder. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.85 (t, J = 5.6 Hz, 1H), 7.60 (s, 1H), 7.51 (s, 2H), 7.41 (d, J = 8.8 Hz, 2H), 7.37 (d, J = 9.0 Hz, 2H), 7.35 (d, J = 8.4 Hz, 2H), 7.26 (d, J = 8.4 Hz, 2H), 3.40 (q, J = 7.1 Hz, 2H), 2.80 (t, J = 7.3 Hz, 2H). ^{13}C NMR (126 MHz, DMSO) δ 165.22, 161.33, 138.72, 133.22, 130.73, 130.57, 129.97, 129.01, 128.94, 128.28, 125.08, 119.91, 108.13, 40.11, 34.73. MS (ESI): m/z 391.10 [M+H]$^+$.

2-Amino-N,4-dibutyl-5-(4-chlorophenyl)thiophene-3-carboxamide, 3d. 1-(4-chlorophenyl)hexan-2-one (**1c**, 0.2 g, 1 mmol) and 2-(4-chlorophenyl)acetaldehyde (**1a**, 0.15 g, 1 mmol) were dissolved in dry THF (5 mL). To the stirred solution, $TiCl_4$ (0.22 mL, 2.0 mmol) was added dropwise, followed by addition of Et_3N (0.3 mL). The reaction mixture was stirred overnight at 40 °C, followed by the addition of 1 N HCl (25 mL) and then extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with 1 N NaOH (25 mL), dried over MgSO4, filtrated and the solvent was removed under reduced pressure. The resulting mixture was dissolved in EtOH (5 mL), S_8 (32mg, 1.0 mmol) and Et_3N (0.2 mL) were added. The reaction mixture was refluxed overnight. Then, the mixture was diluted with EtOAc (25 mL) and washed with water (2 × 50 mL) and brine (2 × 50 mL). The organic layers were dried over $MgSO_4$, filtrated and the solvent was removed under reduced pressure. The product was obtained as brown solid (151 mg, yield 41%) after flash chromatography with petroleum ether:EtOAc 30:1 (*v/v*) as eluent. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.64 (t, *J* = 5.6 Hz, 1H), 7.45 (d, *J* = 8.5 Hz, 2H), 7.28 (d, *J* = 8.5 Hz, 2H), 6.21 (s, 2H), 3.19 (q, *J* = 6.8 Hz, 2H), 2.66 – 2.58 (m, 2H), 1.49 – 1.43 (m, 2H), 1.33 (dt, *J* = 14.9, 7.2 Hz, 4H), 1.16 (q, *J* = 7.3 Hz, 2H), 0.89 (t, *J* = 7.3 Hz, 3H), 0.76 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 165.47, 155.55, 135.69, 133.52, 131.11, 130.51, 128.70, 117.17, 113.51, 38.36, 32.22, 31.39, 27.12, 22.10, 19.79, 13.79, 13.65. HRMS, calculated for $C_{19}H_{26}ON_2ClS$ $[M + H]^+$: 365.1449, found 365.1449.

2-Amino-4-methyl-N-(naphthalen-1-ylmethyl)-5-(3-(trifluoromethyl)phenyl)thiophene-3-carboxamide, 3i. 1-(3-(trifluoromethyl)phenyl)propan-2-on (**1e**, 0.3 g, 1.5 mmol) and 2-cyano-N-(naphthalen-1-ylmethyl)acetamide (**2h**, 0.3 g, 1.5 mmol) were reacted using a procedure similar to the synthesis of **3d**, affording compound **3i** (178 mg, yield 28%) as brown solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.08 (d, *J* = 8.3 Hz, 1H), 7.89 (d, *J* = 8.0 Hz, 1H), 7.83 (d, *J* = 8.1 Hz, 1H), 7.58 – 7.42 (m, 8H), 6.10 (s, 1H), 5.07 (d, *J* = 5.0 Hz, 2H), 2.16 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.17, 134.95, 134.07, 133.57, 132.87, 131.49, 131.14, 130.88, 129.11, 129.03, 128.89, 128.84, 126.81, 126.27, 126.24, 126.18, 125.61, 123.76, 123.73, 123.53, 119.84, 41.95, 16.20. HRMS, calculated for $C_{24}H_{20}ON_2F_3S$ $[M + H]^+$: 441.1243, found 441.1240.

(R)-2-Amino-4-methyl-N-(1-(naphthalen-1-yl)ethyl)-5-(3-(trifluoromethyl)phenyl)thiophene-3-carboxamide, 3j. 1-(3-(trifluoromethyl)phenyl)propan-2-on (**1e**, 0.4 g, 2 mmol) and (R)-2-cyano-N-(1-(naphthalen-1-yl)ethyl)acetamide (**2i**, 0.5 g, 2 mmol) were reacted using a procedure similar to the synthesis of **3d**, affording compound **3j** (146 mg, yield 16%) as brown solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.18 (d, *J* = 8.5 Hz, 1H), 7.88 (d, *J* = 8.1 Hz, 1H), 7.81 (d, *J* = 8.2 Hz, 1H), 7.59 – 7.43 (m, 8H), 6.19 – 6.02 (m, 2H), 2.19 (s, 3H), 1.78 (d, *J* = 5.9 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.41, 138.60, 134.99, 134.16, 132.87, 131.16, 131.08, 130.90, 129.14, 129.04, 128.88, 128.53, 126.73, 126.29, 126.26, 126.02, 125.44, 123.76, 123.74, 123.49, 122.97, 122.69, 45.04, 21.35, 16.23. HRMS, calculated for $C_{25}H_{22}ON_2F_3S$ $[M + H]^+$: 455.1399, found 455.1397.

(S)-2-Amino-4-methyl-N-(1-(naphthalen-1-yl)ethyl)-5-(3-(trifluoromethyl)phenyl)thiophene-3-carboxamide, 3k. 1-(3-(trifluoromethyl)phenyl)propan-2-on (**1e**, 0.2 g, 1 mmol) and (S)-2-cyano-N-(1-(naphthalen-1-yl)ethyl)acetamide (**2i**, 0.2 g, 1 mmol) were reacted using a procedure similar to the synthesis of **3d**, affording compound **3k** (0.2 g, yield 42%) as brown solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.19 (d, *J* = 8.5 Hz, 1H), 7.88 (d, *J* = 8.1 Hz, 1H), 7.82 (d, *J* = 8.2 Hz, 1H), 7.63 – 7.39 (m, 9H), 6.13 – 6.07 (m, 1H), 2.19 (s, 3H), 1.81 (m, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.42, 138.63, 135.03, 134.19, 132.88, 131.10, 130.98, 130.92, 129.14, 129.05, 128.89, 128.54, 126.74, 126.31, 126.28, 126.02, 125.45, 123.93, 123.73, 123.50, 122.97, 122.69, 45.06, 21.37, 16.22. HRMS, calculated for $C_{25}H_{22}ON_2F_3S$ $[M + H]^+$: 455.1399, found 455.1398.

2-Acetamido-N-butyl-5-phenylthiophene-3-carboxamide, 4a. To a stirred solution of 2-amino-N-butyl-5-phenylthiophene-3-carboxamide (0.3g, 1.0 mmol) in dimethylformamide (DMF) (4 mL), acyl chloride (2.0 mmol) and pyridine (2 mmol) were added and dissolved. The reaction mixture was stirred overnight at rt. Then, the mixture was diluted with CH_2Cl_2 (20 mL) and was washed with 1 N HCl (20 mL), $NaHCO_3$ (20 mL), water (20 mL) and brine (2 × 20 mL). The organic layer was dried over $MgSO_4$, filtrated and the solvent was removed under reduced pressure. Product was obtained as brown solid (182 mg, yield 58%). ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 12.06 (s, 1H), 8.39 (t, *J* = 5.5 Hz, 1H), 7.88 (s, 1H), 7.57 (d, *J* = 7.3 Hz, 2H), 7.42 (t, *J* = 7.8 Hz, 2H), 7.29 (t, *J* = 7.9 Hz, 1H), 3.28 (q, *J* = 7.0 Hz, 2H), 2.22 (s, 3H), 1.53 (p, *J* = 7.4 Hz, 2H), 1.34 (h, *J* = 7.3 Hz, 2H), 0.91 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 166.99, 164.51, 144.58, 133.62, 131.67, 129.31, 127.31, 124.82, 118.78, 115.85, 38.45, 31.23, 23.22, 19.71, 13.75. HRMS, calculated for $C_{17}H_{21}O_2N_2S$ $[M + H]^+$: 317.1318, found 317.1317.

N-butyl-2-butyramido-5-phenylthiophene-3-carboxamide, 4b. **4b** was prepared by following the similar procedure for the synthesis of **4a**. Product was obtained as brown solid (152 mg, yield 44%). **^{1}H NMR** (500 MHz, DMSO-*d*$_6$) *δ* 12.2 (s, 1H), 8.4 (t, *J* = 5.7 Hz, 1H), 7.9 (s, 1H), 7.6 – 7.5 (m, 2H), 7.4 (t, *J* = 7.7 Hz, 2H), 7.3 (td, *J* = 7.3, 1.1 Hz, 1H), 3.3 (td, *J* = 7.1, 5.6 Hz, 2H), 2.5 (t, *J* = 7.3 Hz, 2H), 1.6 (h, *J* = 7.3 Hz, 2H), 1.5 (tt, *J* = 7.7, 6.5 Hz, 2H), 1.3 (h, *J* = 7.3 Hz, 2H), 0.9 (dt, *J* = 10.0, 7.3 Hz, 6H) ppm. **^{13}C NMR** (126 MHz, DMSO-*d*$_6$) *δ* 169.6, 164.6, 144.6, 133.6, 131.7, 129.2, 127.3, 124.8, 124.6, 118.8, 118.6, 115.9, 38.5, 37.6, 31.2, 19.7, 18.3, 13.7, 13.5. HRMS, calculated for $C_{19}H_{25}O_2N_2S$ $[M + H]^+$: 345.1631, found 345.1631.

N-butyl-2-heptanamido-5-phenylthiophene-3-carboxamide, 4c. **4c** was prepared by following the similar procedure for the synthesis of **4a**. Product was obtained as brown solid (195 mg, yield 51%). ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 12.15 (s, 1H), 8.39 (t, *J* = 5.6 Hz, 1H), 7.88 (s, 1H), 7.57 (d, *J* = 7.5 Hz, 2H), 7.43 (t, *J* = 7.8 Hz, 2H), 7.29 (t, *J* = 7.4 Hz, 1H), 2.49 – 2.45 (m, 2H), 2.18 (t, *J* = 7.4 Hz, 2H), 1.61 (p, *J* = 7.2 Hz, 2H), 1.53 (p, *J* = 7.4 Hz, 2H), 1.35 (dt, *J* = 14.8, 7.4 Hz, 2H), 1.27 – 1.23 (m, 8H), 0.91 (t, *J* = 7.4 Hz, 3H), 0.85 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 174.98, 170.15, 165.03, 145.02, 134.09, 132.11, 129.67, 127.74, 125.06, 116.31, 38.90, 36.15, 34.12, 31.62, 28.94, 25.23, 24.97, 22.53, 20.15, 14.42, 14.19. HRMS, calculated for $C_{23}H_{33}O_2N_2S$ [M + H]$^+$: 401.2255, found 401.2254.

3-Butyl-6-phenylthieno[2,3-d]pyrimidine-2,4(1H,3H)-dione, 5a. To a stirred solution of 2-amino-N-butyl-5-phenylthiophene-3-carboxamide (0.3g, 1.0 mmol) in DCM (10 mL), 1,1-Carbonyldiimidazole (CDI, 0.5g, 3.0 mmol) was added and the reaction mixture was refluxed for 16 h. Subsequently, the solvent was removed under reduce pressure and EtOAc (25 mL) was added to dissolve the residual mixture. The organic solution was then washed with water (25 mL) and brine (25 mL). The organic layer was collected and dried over $MgSO_4$. After the removal of $MgSO_4$ by filtration, the solvent was removed under reduced pressure. After purified using flash chromatography with petroleum ether:EtOAc 3:1 (*v/v*) as eluent, 159 mg light-yellow solid was obtained as product. Yield 52%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.66 (d, J = 7.4 Hz, 2H), 7.58 (s, 1H), 7.41 (t, J = 7.7 Hz, 2H), 7.32 (t, J = 7.4 Hz, 1H), 3.96 – 3.73 (m, 2H), 1.53 (p, J = 7.5 Hz, 2H), 1.30 (h, J = 7.4 Hz, 2H), 0.90 (t, J = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 158.32, 150.26, 149.70, 133.44, 132.60, 129.25, 127.95, 125.29, 118.10, 115.60, 29.48, 22.39, 19.68, 13.76. HRMS, calculated for $C_{16}H_{17}O_2N_2$ [M + H]$^+$: 301,1005, found 301,1006.

3-Allyl-6-(4-chlorophenyl)thieno[2,3-d]pyrimidine-2,4(1H,3H)-dione, 5b. N-allyl-2-amino-5-(4-chlorophenyl)thiophene-3-carboxamide (0.3 g, 1 mmol) was reacted with CDI (0.5g, 3.0 mmol) following a similar method for synthesis of **5a** to provide **5b** as 126 mg light-yellow solid as product. Yield 40%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.39 (s, 1H), 7.69 (d, J = 8.4 Hz, 2H), 7.65 (s, 1H), 7.46 (d, J = 8.4 Hz, 2H), 5.85 (dq, J = 16.0, 5.2 Hz, 1H), 5.14 – 5.05 (m, 2H), 4.45 (d, J = 4.5 Hz, 2H). ^{13}C NMR (126 MHz, DMSO) δ 157.93, 150.19, 149.97, 132.71, 132.33, 132.11, 131.53, 129.14, 126.90, 118.89, 116.23, 115.55, 41.93. MS (ESI): m/z 319.08 [M+H]$^+$.

5-Methyl-3-(naphthalen-1-ylmethyl)-6-(3-(trifluoromethyl)phenyl)thieno[2,3-d]pyrimidine-2,4(1H,3H)-dione, 5c. **3i** (50 mg, 0.1 mmol) and CDI (50 mg, 0.3 mmol) were reacted using a procedure similar to the synthesis of **5a**, affording compound **5c** (48 mg, yield 91%) as brown solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.56 (s, 1H), 8.22 (d, J = 8.3 Hz, 1H), 7.98 (d, J = 8.0 Hz, 1H), 7.84 – 7.72 (m, 5H), 7.64 – 7.56 (m, 2H), 7.42 (t, J = 7.7 Hz, 1H), 7.10 (d, J = 7.2 Hz, 1H), 5.53 (s, 2H), 2.44 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 159.26, 151.23, 150.24, 133.49, 133.22, 132.22, 131.25, 130.39, 130.19, 129.90, 129.65, 128.70, 127.23, 127.07, 126.44, 126.26, 126.04, 125.85, 125.51, 125.09, 123.00, 121.97, 113.80, 40.92, 14.10. HRMS, calculated for $C_{25}H_{18}O_2N_2F_3S$ [M + H]$^+$: 467,1036, found 467,1034.

(R)-5-Methyl-3-(1-(naphthalen-1-yl)ethyl)-6-(3-(trifluoromethyl)phenyl)thieno[2,3-d]pyrimidine-2,4(1H,3H)-dione, 5d. **3j** (0.1 g, 0.2 mmol) and CDI (0.2 g, 1.2 mmol) were reacted using a procedure similar to the synthesis of **5a**, affording compound **5d** (68 mg, yield 64%) as brown solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 11.27 (s, 1H), 7.98 (dd, *J* = 10.4, 7.9 Hz, 2H), 7.80 (d, *J* = 8.3 Hz, 1H), 7.76 (d, *J* = 8.2 Hz, 1H), 7.65 (t, *J* = 3.6 Hz, 2H), 7.62 – 7.57 (m, 2H), 7.53 – 7.49 (m, 1H), 7.46 – 7.37 (m, 2H), 6.91 (q, *J* = 7.0 Hz, 1H), 2.56 (s, 3H), 2.03 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 159.50, 152.27, 150.01, 134.73, 133.75, 133.72, 132.92, 132.32, 131.79, 129.51, 129.08, 128.40, 127.93, 126.64, 126.36, 126.33, 126.26, 125.34, 124.99, 124.84, 124.81, 123.20, 115.32, 47.92, 16.87, 14.65. HRMS, calculated for $C_{26}H_{20}O_2N_2F_3S$ $[M + H]^+$: 481,1192, found 481,1192.

(S)-5-Methyl-3-(1-(naphthalen-1-yl)ethyl)-6-(3-(trifluoromethyl)phenyl)thieno[2,3-d]pyrimidine-2,4(1H,3H)-dione, 5e. **3k** (0.1 g, 0.2 mmol) and CDI (0.2 g, 1.2 mmol) were reacted using a procedure similar to the synthesis of **5e**, affording compound **5d** (82 mg, yield 77%) as light-yellow solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 11.13 (s, 1H), 7.97 (m, 2H), 7.81 (d, *J* = 7.8 Hz, 1H), 7.76 (d, *J* = 8.2 Hz, 1H), 7.64 (d, *J* = 5.4 Hz, 2H), 7.59 (d, *J* = 3.5 Hz, 2H), 7.51 (t, *J* = 7.7 Hz, 1H), 7.42 (dt, *J* = 22.1, 7.4 Hz, 2H), 6.91 (q, *J* = 6.6 Hz, 1H), 2.55 (s, 3H), 2.03 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 159.53, 152.09, 150.00, 134.81, 133.77, 133.74, 132.94, 132.33, 131.81, 129.50, 129.08, 128.40, 127.89, 126.65, 126.37, 126.34, 126.27, 125.35, 124.98, 124.83, 124.81, 123.23, 115.32, 47.93, 16.89, 14.64. HRMS, calculated for $C_{26}H_{20}O_2N_2F_3S$ $[M + H]^+$: 481,1192, found 481,1192.

Ethyl 2-amino-4-methyl-5-phenylthiophene-3-carboxylate, 6a. A mixture of phenolacetone (**1f**, 0.7 mL, 5 mmol), ethyl cyanoacetate (0.5 mL, 5mmol), ammonium acetate (0.1 g, 1 mmol), and acetic acid (0.2 mL, 4 mmol) in toluene (5 mL) was heated under reflux for 20 h, while water was removed using molecular sieve. After the mixture was cooled to room temperature, the mixture was concentrated in vacuo. The residue was diluted with saturated $NaHCO_3$ (20 mL) and extracted with $CHCl_3$ (3 × 25 mL). The extract was washed with brine (25 mL) and dried ($MgSO_4$). After evaporation of the solvent in vacuo, the residue was purified by flash column chromatography to give an oil-like intermediate, which was dissolved in EtOH (9 mL). To the solution were added sulfur powder (130 mg, 4 mmol) and triethylamine (0.6 mL, 4 mmol), and the resulting mixture was stirred at 100 °C for 2 h. After removal of the solvent in vacuo, the residue was diluted with brine and extracted with $CHCl_3$ (3 × 25 mL). The organic layers was collected and washed with brine (25 mL) and dried ($MgSO_4$). The solution was concentrated in vacuo, and the residue was recrystallized from $CHCl_3$ to afford **6a** (1.1 g, 81%) as dark-brown crystals. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.82

(t, J = 5.8 Hz, 1H), 7.46 (s, 2H), 7.40 (d, J = 8.0 Hz, 2H), 7.34 (t, J = 7.8 Hz, 2H), 4.36 (q, J = 7.0 Hz, 3H), 1.34 (t, J = 7.1 Hz, 3H).

Ethyl 2-amino-5-(4-chlorophenyl)-4-methylthiophene-3-carboxylate, 6b. 4-chlorophenylacetone (**1j**, 0.7mL, 5 mmol) and ethyl cyanoacetate (0.5 g, 5 mmol) were reacted using a procedure similar to the synthesis of **6a**, affording compound **6b** (0.9 g, yield 61%) as brown solid. ^{1}H NMR (500 MHz, Chloroform-d) δ 7.34 (d, J = 8.5 Hz, 2H), 7.27 (d, J = 7.3 Hz, 2H), 6.12 (s, 2H), 4.35 – 4.28 (m, 2H), 2.30 (s, 3H), 1.37 (t, J = 7.1 Hz, 3H).

Ethyl 2-amino-5-(3,4-dichlorophenyl)-4-methylthiophene-3-carboxylate, 6c. 1-(3,4-dichlorophenyl)propan-2-one (**1k**, 0.3 mL, 2 mmol) and ethyl cyanoacetate (0.5 g, 5 mmol) were reacted using a procedure similar to the synthesis of **6a**, affording compound **6c** (0.3 g, yield 39%) as brown solid. ^{1}H NMR (500 MHz, Methanol-d_4) δ 7.54 (s, 1H), 7.29 (d, J = 7.1 Hz, 1H), 7.27 (d, J = 7.1 Hz, 1H), 4.13 (q, J = 7.0 Hz, 32), 2.05 (s, 3H), 1.27 (t, J = 7.1 Hz, 3H).

Ethyl 2-amino-5-(2,4-dichlorophenyl)-4-methylthiophene-3-carboxylate, 6d. 1-(2,4-dichlorophenyl)propan-2-one (**1l**, 0.3 mL, 2 mmol) and ethyl cyanoacetate (0.3 g, 3 mmol) were reacted using a procedure similar to the synthesis of **6a**, affording compound **6d** (0.3 g, yield 49%) as brown solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.72 (d, J = 2.1 Hz, 1H), 7.49 – 7.38 (m, 4H), 4.20 (q, J = 7.1 Hz, 2H), 1.99 (s, 3H), 1.27 (t, J = 7.1 Hz, 3H).

Ethyl 2-amino-4-methyl-5-(3-(trifluoromethyl)phenyl)thiophene-3-carboxylate, 6e. **1e** (0.7mL, 5 mmol) and ethyl cyanoacetate (0.5 g, 5 mmol) were reacted using a procedure similar to the synthesis of **6a**, affording compound **6e** (1.0 g, yield 68 %) as brown solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.64 (m, 3H), 7.58 (s, 1H), 7.55 (s, 2H), 4.22 (q, J = 7.1 Hz, 2H), 2.26 (s, 3H), 1.28 (t, J = 7.1 Hz, 3H).

Ethyl 2-amino-5-(benzo[d][1,3]dioxol-5-yl)-4-methylthiophene-3-carboxylate, 6f. 1-(benzo[d][1,3]dioxol-5-yl)propan-2-one (**1m**, 0.3 mL, 2 mmol) and ethyl cyanoacetate (0.5 g, 5 mmol) were reacted using a procedure similar to the synthesis of 6a, affording compound 6b (0.2 g, yield 28%) as brown solid. ^{1}H NMR (500 MHz, Chloroform-d) δ 7.74 (s, 1H), 7.44 (d, J = 6.8 Hz, 1H), 6.94 (d, J = 6.1 Hz, 1H), 6.84 (s, 2H), 6.12 (s, 2H), 4.39 (q, J = 6.8 Hz, 2H), 2.31 (s, 3H), 1.40 (t, J = 6.5 Hz, 3H).

Ethyl 2-amino-5-(4-fluorophenyl)-4-methylthiophene-3-carboxylate, 6g. 1-(benzo[d][1,3]dioxol-5-yl)propan-2-one (**1m**, 0.7mL, 5 mmol) and ethyl cyanoacetate (0.5 g, 5 mmol) were reacted using a procedure similar to the synthesis of 6a, affording compound 6b (0.8 g, yield 59%) as brown solid. 1H NMR (500 MHz, Methanol-d4) δ 7.37 – 7.31 (m, 2H), 7.14 – 7.10 (m, 2H), 4.30 (q, J = 7.1 Hz, 2H), 2.27 (s, 3H), 1.37 (t, J = 7.1 Hz, 3H).

5-Methyl-3,6-diphenylthieno[2,3-d]pyrimidine-2,4(1H,3H)-dione, 7a. To a stirred solution of **6a** (0.3 g, 1 mmol) in pyridine (3 mL), phenyl isocyanate (0.2 mL, 1.5 mmol) was added and stirred at 45 °C for overnight. The resulting mixture was concentrated reduced pressure and the residue was suspended in MeOH (5 mL). To the suspension, 25% sodium methoxide (0.6 mL, 3 mmol) was added and stirred for 6 hours at room temperature. Subsequently, the reaction mixture was acidified with 1N HCl (10 ml) at 0 °C and the pH was regulated to 4. The organic solvent was evaporated and the precipitate was collected by filtration. Obtained solid was dissolved in methanol and ourified with flash chromatography using DCM:MeOH (100:1) as solvent. The product was obtained as light-brown crystals (135 mg, yield 40%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.37 (s, 1H), 7.51 – 7.39 (m, 8H), 7.31 – 7.27 (m, 2H), 2.40 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 159.44, 150.86, 150.15, 135.79, 132.38, 129.83, 129.22, 129.07, 128.99, 128.86, 128.71, 127.97, 126.74, 114.19, 14.15. HRMS, calculated for $C_{19}H_{15}O_2N_2S$ [M + H]$^+$: 335.0849, found 335.0849.

3-Benzyl-5-methyl-6-phenylthieno[2,3-d]pyrimidine-2,4(1H,3H)-dione, 7b. **6a** (0.3 g, 1 mmol) and benzyl isocyanate (0.2 mL, 1.5 mmol) were reacted using a procedure similar to the synthesis of **7a**, affording compound **7b** (150 mg, yield 43%) as white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.36 (s, 1H), 7.49 – 7.44 (m, 4H), 7.39 (t, *J* = 6.8 Hz, 1H), 7.32 – 7.30 (m, 4H), 7.25 – 7.23 (m, 1H), 5.04 (s, 2H), 2.43 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 159.19, 150.43, 150.20, 137.49, 132.34, 129.65, 129.16, 129.08, 128.34, 127.97, 127.40, 127.04, 126.86, 113.76, 42.89, 14.15. HRMS, calculated for $C_{20}H_{17}O_2N_2S$ [M + H]$^+$: 349.1005, found 349.1004.

6-(4-Chlorophenyl)-5-methyl-3-phenylthieno[2,3-d]pyrimidine-2,4(1H,3H)-dione, 7c. **6b** (0.3 g, 1 mmol) and phenyl isocyanate (0.2 mL, 1.5 mmol) were reacted using a procedure similar to the synthesis of **7a**, affording compound **7c** (93 mg, yield 31%) as white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.40 (s, 1H), 7.54 (d, *J* = 8.6 Hz, 2H), 7.51 – 7.46 (m, 4H), 7.41 (t, *J* = 7.4 Hz, 1H), 7.29 (d, *J* = 7.1 Hz, 2H), 2.40 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 159.84, 151.48, 150.55, 136.19, 133.12, 131.73, 131.40, 131.23, 131.12, 131.00, 129.65, 129.37, 125.80, 114.64, 14.55. MS (ESI): m/z 369.09 [M + H]$^+$.

3-Benzyl-6-(4-chlorophenyl)-5-methylthieno[2,3-d]pyrimidine-2,4(1H,3H)-dione, 7d. **6b** (0.2 g, 0.7 mmol) and benzyl isocyanate (0.2 mL, 1.5 mmol) were reacted using a procedure similar to the synthesis of **7a**, affording compound **7d** (73 mg, yield 34%) as white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.38 (s, 1H), 7.53 (d, J = 6.1 Hz, 2H), 7.47 (d, J = 7.6 Hz, 2H), 7.31 – 7.24 (m, 5H), 5.04 (s, 2H), 2.43 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 159.58, 151.07, 150.60, 137.91, 133.11, 131.70, 131.28, 130.82, 129.50, 128.79, 127.86, 127.49, 125.92, 114.20, 43.36, 14.56. HRMS, calculated for $C_{20}H_{16}O_2N_2ClS$ [M + H]$^+$: 383.0616, found 383.0616.

6-(4-Chlorophenyl)-5-methyl-3-(4-(trifluoromethyl)benzyl)thieno[2,3-d]pyrimidine-2,4(1H,3H)-dione, 7e. **6b** (0.2 g, 0.5 mmol) and 4-(Trifluoromethyl)benzyl isocyanate (0.2 mL, 1.0 mmol) were reacted using a procedure similar to the synthesis of **7a**, affording compound **7e** (0.1 g, yield 44%) as white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.44 (s, 1H), 7.68 (d, J = 6.8 Hz, 2H), 7.56 – 7.44 (m, 6H), 5.12 (s, 2H), 2.42 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 159.57, 151.26, 150.58, 142.72, 133.15, 131.67, 131.28, 129.49, 129.44, 128.45, 126.03, 125.78, 125.65, 114.18, 43.16, 14.52. HRMS, calculated for $C_{21}H_{15}O_2N_2ClF_3S$ [M + H]$^+$: 451.0489, found 451.0489.

3-Benzyl-6-(3,4-dichlorophenyl)-5-methylthieno[2,3-d]pyrimidine-2,4(1H,3H)-dione, 7f. **6c** (0.2 g, 0.5 mmol) and benzyl isocyanate (0.2 mL, 1.5 mmol) were reacted using a procedure similar to the synthesis of **7a**, affording compound **7f** (0.1 g, yield 48%) as white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.34 (s, 1H), 7.53 (dt, J = 5.9, 2.7 Hz, 2H), 7.27 (t, J = 6.2 Hz, 5H), 6.74 (d, J = 4.5 Hz, 1H), 5.00 (s, 2H), 4.16 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 158.91, 150.35, 141.33, 137.47, 136.65, 130.71, 130.66, 130.41, 129.23, 128.66, 128.31, 127.47, 127.07, 120.90, 113.57, 111.82, 42.77, 33.69. HRMS, calculated for $C_{20}H_{15}O_2N_2Cl_2S$ [M + H]$^+$: 417.0226, found 417.0225.

3-Benzyl-6-(2,4-dichlorophenyl)-5-methylthieno[2,3-d]pyrimidine-2,4(1H,3H)-dione, 7g. **6d** (0.2 g, 0.5 mmol) and benzyl isocyanate (0.2 mL, 1.5 mmol) were reacted using a procedure similar to the synthesis of **7a**, affording compound **7g** (82 mg, yield 39%) as white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.37 (s, 1H), 7.81 (d, J = 2.0 Hz, 1H), 7.56 – 7.49 (m, 2H), 7.33 – 7.29 (m, 4H), 7.26 – 7.23 (m, 1H), 5.04 (s, 2H), 2.18 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 159.49, 151.85, 150.66, 137.91, 135.49, 135.01, 134.92, 133.15, 130.05, 129.79, 128.81, 127.91, 127.88, 127.53, 122.38, 113.26, 43.39, 14.68. HRMS, calculated for $C_{20}H_{15}O_2N_2Cl_2S$ [M + H]$^+$: 417.0226, found 417.0225.

3-Benzyl-5-methyl-6-(3-(trifluoromethyl)phenyl)thieno[2,3-d]pyrimidine-2,4(1H,3H)-dione, 7h. **6e** (0.2 g, 0.5 mmol) and benzyl isocyanate (0.2 mL, 1.5 mmol) were reacted using a procedure similar to the synthesis of **7a**, affording compound **7h** (130 mg, yield 62%) as white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.46 (s, 1H), 7.75 (m, 4H), 7.32 (m, 4H), 7.26 (m, 1H), 5.06 (s, 2H), 2.45 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 159.17, 151.01, 150.19, 137.45, 133.49, 133.24, 131.20, 130.37, 129.87, 129.62, 128.38, 127.43, 127.10, 125.25, 124.96, 122.89, 113.74, 42.94, 14.10. HRMS, calculated for $C_{21}H_{16}O_2N_2F_3S$ [M + H]$^+$: 417.0879, found 417.0879.

6-(Benzo[d][1,3]dioxol-5-yl)-3-benzyl-5-methylthieno[2,3-d]pyrimidine-2,4(1H,3H)-dione, 7i. **6f** (0.2 g, 0.5 mmol) and benzyl isocyanate (0.2 mL, 1.5 mmol) were reacted using a procedure similar to the synthesis of **7a**, affording compound **7i** (38 mg, yield 20%) as white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.33 (s, 1H), 7.27 (dd, J = 19.8, 6.8 Hz, 5H), 7.01 (dd, J = 10.2, 5.7 Hz, 2H), 6.90 (t, J = 6.3 Hz, 1H), 6.08 (s, 2H), 5.04 (s, 2H), 2.39 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 159.61, 150.67, 150.49, 148.16, 147.62, 137.95, 129.63, 128.80, 127.81, 127.48, 127.19, 126.31, 123.60, 114.03, 109.95, 109.19, 101.92, 43.31, 14.54. HRMS, calculated for $C_{21}H_{17}O_4N_2S$ [M + H]$^+$: 393.0904, found 393.0897.

2-(Chloromethyl)-6-(4-chlorophenyl)-5-methylthieno[2,3-d]pyrimidin-4(1H)-one, 8a. **6b** (0.3 g, 1 mmol) and 2-chloroacetonitrile (0.1 mL, 1.2 mmol) were diluted into 4N HCl in 1,4-dioxane (2 mL) and stirred for 1h at room temperature. Subsequently, the reaction mixture was heated to 100°C 12h until precipitation formed. After cooled down to room temperature, the reaction mixture was filtered, and the precipitate was washed with *n*-hexane. The final product was purified with column chromatography with DCM:MeOH 10:1 (*v/v*). 195 mg white solid was obtained as product. Yield 60%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.81 (s, 1H), 7.55 (m, 4H), 4.57 (s, 2H), 2.53 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 162.8, 158.8, 153.1, 133.1, 132.4, 131.5, 131.1, 130.9, 130.0, 129.1, 128.9, 123.0, 42.5, 14.3. HRMS, calculated for $C_{14}H_{11}ON_2Cl_2S$ [M + H]$^+$: 324.9964, found 324.9964.

2-Benzyl-6-(4-chlorophenyl)-5-methylthieno[2,3-d]pyrimidin-4(1H)-one, 8b. **6b** (0.3 g, 1 mmol) was reacted with 2-phenylacetonitrile (0.2 g, 1.6 mmol) following similar procedure of synthesis of **8a** to afford **8b** (201 mg, 55%) as a white solid; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.6 (s, 1H), 7.6 – 7.5 (m, 4H), 7.4 – 7.3 (m, 4H), 7.3 – 7.2 (m, 1H), 4.0 (s, 2H), 2.5 (s, 3H) ppm. ^{13}C NMR (126 MHz, DMSO-d_6) δ 163.8, 159.1, 157.1, 136.3, 132.9, 131.7, 131.0, 130.9, 130.8, 130.7, 129.7, 129.1, 128.9, 128.6, 126.9, 121.9 56.9, 26.6 ppm. HRMS, calculated for $C_{20}H_{16}ON_2ClS$ [M + H]$^+$: 367.0666, found 367.0665.

2-Benzyl-6-(4-fluorophenyl)-5-methylthieno[2,3-d]pyrimidin-4(1H)-one, 8c. **6g** (0.3 g, 1 mmol) was reacted with 2-(3-methoxyphenyl)acetonitrile (0.2 g, 1.4 mmol) following similar procedure of synthesis of **8a** to afford **8c** (127 mg, 32%) as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.65 (s, 1H), 7.53 (dd, J = 8.7, 5.4 Hz, 2H), 7.37 – 7.29 (m, 6H), 7.25 (t, J = 6.7 Hz, 1H), 3.95 (s, 2H), 2.49 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 164.1, 161.3, 159.7, 157.4, 136.82, 131.9, 131.5, 129.7, 129.6, 129.4, 129.0, 127.4, 122.3, 116.3, 40.6, 14.7. HRMS, calculated for $C_{20}H_{16}ON_2FS$ [M + H]$^+$: 351.0962, found 351.0961.

6-(4-Chlorophenyl)-2-(3-methoxybenzyl)-5-methylthieno[2,3-d]pyrimidin-4(1H)-one, 8d. **6g** (0.3 g, 1 mmol) was reacted with 2-phenylacetonitrile (0.2 g, 1.6 mmol) following similar procedure of synthesis of **8a** to afford **8d** (203 mg, 53%) as a white solid. **^{1}H NMR** (500 MHz, DMSO-d_6) *δ* 12.6 (s, 1H), 7.6 – 7.4 (m, 4H), 7.2 (t, J = 7.8 Hz, 1H), 7.0 – 6.9 (m, 1H), 6.9 (d, J = 7.8 Hz, 1H), 6.8 (ddd, J = 8.4, 2.7, 1.0 Hz, 1H), 3.9 (s, 2H), 3.7 (s, 3H), 2.5 (s, 3H) ppm. **^{13}C NMR** (126 MHz, DMSO-d_6) *δ* 163.8, 159.3, 159.1, 156.9, 137.7, 132.8, 131.7, 131.0, 130.8, 130.7, 129.7, 129.6, 129.0, 128.8, 121.9, 121.0, 114.8, 112.2, 55.1, 54.9, 14.2. HRMS, calculated for $C_{21}H_{18}O_2N_2ClS$ [M + H]$^+$: 397.0772, found 397.0770.

Ethyl 2-amino-4-phenylthiophene-3-carboxylate, ***10a***. An equimolar mixture of powdered sulfur (160 mg, 5mmol) and morpholine (0.5 mL) was stirred until total dissolution of the sulfur. After, the ethyl cyanoacetate (0.6 mL, 5 mmol) and the acetophenone (0.6 mL, 5 mmol) were added to the reactional mixture, which was stirred at room temperature for 18 h. After completion of the reaction, as monitored by TLC, the crude product was chromatographed on silica with CH_2Cl_2 to afford white solid, yield 34%.

Ethyl 2-amino-4-(4-chlorophenyl)thiophene-3-carboxylate, 10b. **10b** was synthesized by following the same method as the preparation of 10a using 1-(4-chlorophenyl)ethan-1-one (5 mmol) as starting material. 250 mg white solid was obtained as product with a yield of 18%.

3-Benzyl-5-phenylthieno[2,3-d]pyrimidine-2,4(1H,3H)-dione, 11a. **11a** was prepared similarly as **7a** with a yield of 67%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.43 (s, 1H), 7.44 (d, J = 6.4 Hz, 2H), 7.35 (q, J = 8.2, 7.3 Hz, 3H), 7.27 (d, J = 6.5 Hz, 4H), 7.21 (t, J = 6.4 Hz, 1H), 7.04 (s, 1H), 5.00 (s, 2H). ^{13}C NMR (126 MHz, DMSO) δ 158.14, 152.69, 150.33, 138.92, 137.51, 134.95, 129.28, 128.32, 127.55, 127.47, 127.40, 127.01, 114.74, 111.16, 43.06. HRMS, calculated for $C_{19}H_{15}O_2N_2S$ [M + H]$^+$: 335.0849, found 335.0848.

3-Benzyl-5-(4-chlorophenyl)thieno[2,3-d]pyrimidine-2,4(1H,3H)-dione, **11b**. **11b** was prepared similarly as **7a** with a yield of 56%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.42 (s, 1H), 7.46 (d, J = 8.4 Hz, 2H), 7.42 (d, J = 8.6 Hz, 2H), 7.31 – 7.19 (m, 5H), 7.08 (s, 1H), 5.00 (s, 2H). ^{13}C NMR (126 MHz, DMSO) δ 158.13, 152.72, 150.22, 137.43, 137.39, 133.68, 132.26, 130.94, 128.24, 127.45, 127.35, 126.94, 121.99, 115.13, 43.03. HRMS, calculated for $C_{19}H_{14}O_2N_2ClS$ [M + H]$^+$: 369.0459, found 369.0459.

Preparation of recombinant human MIF2. Detailed procedures to produce recombinant human MIF2 were reported in Song et al., *EBioMedicine* **2021**, *68*, 103412. Gene sequences of the human MIF2 gene (Invitrogen) was adapted to bacterial expression. After subcloning into a pET20b(+) expression vector, IPTG-induced expression was performed in *E.coli* strain BL21(DE3). MIF2 proteins were overproduced overnight and harvested cells were resuspended and sonicated. The soluble fraction was purified using a Q sepharose column (GE Healthcare) with a gradient of NaCl. The fractions containing MIF2 were brought to 1.7 M ammonium sulfate and loaded on a phenyl sepharose column (GE Healthcare) and eluted with a gradient to 0 M ammonium sulfate in a 20 mM sodium phosphate buffer, pH 8.0. Finally, the proteins were purified by size exclusion chromatography on a Superdex75 column (GE Healthcare) in 20 mM sodium phosphate buffer, pH 8.0, with an elution volume characteristic for trimeric MIF2. The collected protein was concentrated using a VivaSpin centrifugation column with a molecular weight cut off at 5000 Da (Sartorius Stedim Biotech GmbH). Purified proteins were aliquoted, snap frozen in liquid nitrogen, and stored at –80 °C. Purity of obtained protein was tested by SDS-PAGE and coomassie staining.

Optimization of MIF2-catalyzed tautomerization assay. To facilitate the effective assessment of binding potency of MIF2 inhibitors, a convenient and reliable assay was needed. The most widely used assay for MIF inhibitor evaluation is the 4-HPP based tautomerization assay, in which the potency on inhibition of MIF-catalyzed 4-HPP tautomerization is applied to reflect the binding affinity of tested compound to MIF (Ouertatani-Sakouhi et al., *J. Biomol. Screen.* **2010**, *15*, 347–358). This assay was also applied to assess MIF2 binder in previous studies (Tilstam et al., *J. Biol. Chem.* **2019**, *294*, 18522–18531). However, the catalytic activity of MIF2 on the 4-HPP keto-enol conversion is 10-time less active compared with MIF (Merk et al., *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 577–585). Considering that the keto-enol tautomerization of 4-HPP is also a spontaneous process in boric acid buffer, the low catalytic activity of MIF2 makes the MIF2 mediated 4-HPP tautomerization assay a nonoptimal one for MIF2 binding study. Therefore, a more convenient and reliable assay for assessment of MIF2 binding was needed.
The structural features of tautomerase active site of MIF2 were investigated to understand the basis of its activity. Superimposition of the crystal structure of MIF2 (Pantouris et al., *Biochemistry* **2018**, *57*, 3599–3605) to a complex (Cho et al., *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 11313–11318) of MIF and 4-HPP showed that the Pro1 of MIF2 well overlaps with the Pro1 of MIF1, which indicates that the catalytic centers of MIF2 and MIF sit at the same

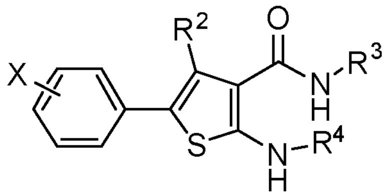
location in the active pockets. Nevertheless, MIF2 losses two hydrogen bonds formed between Asn97 of MIF and the 4-position hydroxyl group of 4-HPP, as the Arg98 locates at the corresponding placement of MIF2. Accordingly, it was hypothesized that MIF2 could exhibit different activity on catalysis the keto-enol tautomerization of 4-HPP analogues with different substituents at 4-position. Therefore, a more active substrate for MIF2 was sought in order to build up a convenient and sensitive enzymatic assay. Three analogues of 4-HPP were synthesized with methoxyl (4-methoxylphenylpyruvate, 4-MPP), chloro (4-chlorophenylpyruvate, 4-CPP), or no substitution (phenylpyruvate, PP) at the 4-position of phenylpyruvate. To investigate the catalytic activity of MIF and MIF2 on these artificial substrates, the keto-enol conversion rate of four substrates by MIF or MIF2 was tested. The measured parameters show that K_M values of MIF on catalyzing PP, 4-CPP, 4-HPP, and 4-MPP keto-enol conversion are 0.76, 0.60, 0.94, and 1.3 mM, respectively. These values are lower than the corresponding values of MIF2, which indicates that MIF has higher binding affinity to all these four substrates than MIF2. However, the catalytic efficiency of MIF and MIF2 on each substrate was diverse. For instance, the catalytic efficiency of MIF on 4-HPP was 7 times higher than MIF2, which can explain the reason why 4-HPP is not a reactive substrate of MIF2. In contrast, MIF2 could catalyze the keto-enol conversion of PP around 3 times more efficient than MIF. Moreover, PP was also the most active substrate of MIF2 among these four tested compounds. Therefore, PP was applied as a replacement of 4-HPP to set up the tautomerase assay for MIF2.

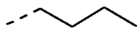
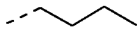
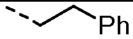
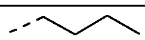
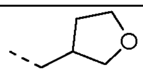
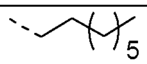
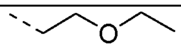
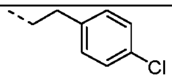
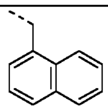
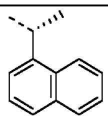
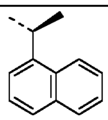
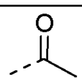
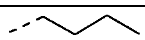
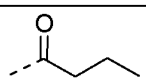
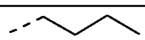
Tautomerase assay. Thus, the focused compound collection described above was tested for inhibition of MIF2 tautomerase activity employing PP as a substrate.
The protocol for measuring MIF tautomerase enzyme activity and enzyme kinetics was described in Xiao et al., *Eur. J. Med. Chem.* **2020**, *186*, 111849–111862. The methods of enzyme study on MIF2 was adapted from the protocol of the MIF study. Briefly, 180 µL of a 500 nM MIF2 solution in boric acid buffer (435 mM, pH 6.2) was mixed with 10 µL of a 20 mM EDTA solution in demiwater and 10 µL of a solution of the desired compound dissolved in DMSO or blank DMSO. This mixture was pre-incubated at room temperature for 10 min. Subsequently, 50 µL of this mixture was mixed with 50 µL of a 1 mM phenylpyruvate (PP) solution in ammonium acetate buffer (50 mM, pH 6.0). MIF2 tautomerase activity was monitored by measuring the increase of UV absorbance at 300 nm over time. MIF2 tautomerase activity in the presence a blank DMSO dilution was set to 100%. Non-catalyzed conversion of the substrate in absence of MIF was set to 0%. Data from the first three minutes were used to calculate the initial velocities. All experiments were repeated three times and the linear regression parameters were determined to calculate the inhibitory potency or the IC_{50} using GraphPad Prism. The window coefficient (*Z'*-factor) of this assay

was proved to be 0.75 in this setup, which indicates the quality of this assay is sufficient for medium- to high-throughput applications (0.5–1) (Zhang et al., *J. Biomol. Screen.* **1999**, *4*, 67–73).

The IC_{50} values of synthesized compounds on inhibition of MIF2 tautomerase activity are shown in Table 1 - 3.

Table 1. Inhibition of the MIF2 tautomerase enzyme activity by thiophene derivatives as determined by the conversion of PP as a substrate. (n = 3, standard deviation of the non-linear curve fitting are reported).



	X	R⁴	R²	R³	IC_{50} (µM)
3a	4-Cl	H	H		15±0.8
3b	4-Br	H	H		7.2±0.6
3c	4-Cl	H	H		84±10
3d	4-Cl	H			15±0.7
3e	H	H	H		168±8.2
3f	H	H	H		13±0.8
3g	4-Cl	H	H		>100
3h	4-Cl	H	H		36±2.7
3i	3-CF_3	H	CH_3		2.6±0.2
3j	3-CF_3	H	CH_3		4.2±0.3
3k	3-CF_3	H	CH_3		7.9±0.4
4a	H		H		41±2.3
4b	H		H		39±1.0

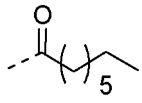
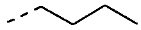
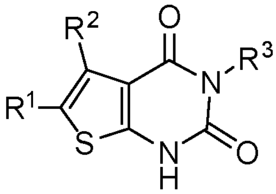
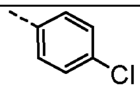
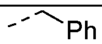
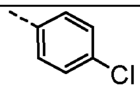
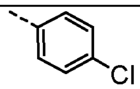
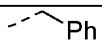
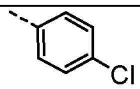
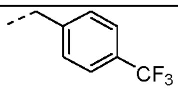
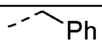
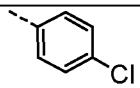
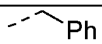
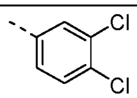
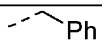
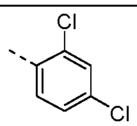
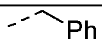
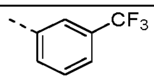
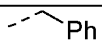
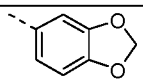
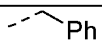
4c	H		H		28±1.3

Table 2. Inhibition of the MIF2 tautomerase enzyme activity by thieno[2,3-*d*]pyrimidine-2,4(1*H*,3*H*)-diones as determined by the conversion of PP as a substrate. (n = 3, standard deviation of the non-linear curve fitting are reported).



	R^2	R^1	R^3	IC_{50} (µM)
5a	H	Ph		16±0.6
5b	H			15±1.0
7a	CH_3	Ph	Ph	>100
7b	CH_3	Ph		7.6±0.5
7c	CH_3		Ph	19±1.0
7d	CH_3			5.1±0.5
7e	CH_3			4.8±0.2
11a	Ph	H		36±3.1
11b		H		27±6.1
7f	CH_3			1.9±0.1
7g	CH_3			3.5±0.3
7h	CH_3			1.7±0.1
7i	CH_3			4.6±0.3

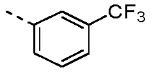
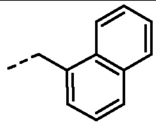
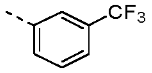
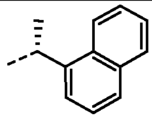
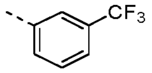
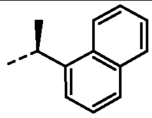
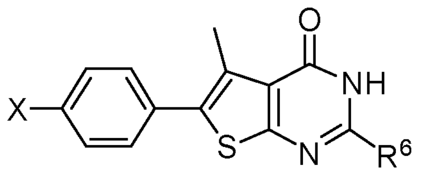
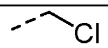
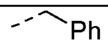
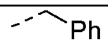
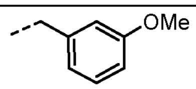
5c	CH_3			0.81±0.1
5d	CH_3			1.0±0.2
5e	CH_3			2.5±0.2

Table 3. Inhibition of the MIF2 tautomerase enzyme activity by thieno[2,3-*d*]pyrimidin-4(1*H*)-ones as determined by the conversion of PP as a substrate. (*n* = 3, standard deviation of the non-linear curve fitting are reported).



	X	R^6	IC_{50} (μM)
8a	Cl		17±1.4
8b	Cl		15±1.4
8c	F		17±1.3
8d	Cl		4.3±0.4

5c has the lowest IC_{50} value. However, the solubility of **5c** is only 3.3 μg/mL (7.4 μM), which would be a limitation for the following cell assays. Both **5d** and **5e** have improved solubility in aqueous solution with saturated concentrations of 16 μg/mL (36 μM) and 15 μg/mL (33 μM) for **5d** and **5e**, respectively. Moreover, **5d** exhibits a comparable potency as **5c**, while **5e** is less active.

The toxicity of **5d** was investigated using an MTS assay, which indicated that **5d** did not inhibit cell viability from a concentrations of 10 μM or lower for a treatment of 24 hours.

Cell culture. Four different human lung cancer cell lines including A549 (ATCC-CCL-185), H1650 (ATCC-CRL-5883), H1299 (ATCC-CRL-5803), and HCC827 (ATCC-CRL-2868) were cultured in RPMI 1640 Medium (GibcoTM #61870-010) containing 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin/streptomycin (GibcoTM#10378016) at 37°C with 5% CO_2 in humidified air.

Cell proliferation assay. Cell proliferation was measured with the CyQUANT® Direct Cell Proliferation Assay Kit (Thermo Fisher, #C35011) by following the protocol. Cells were cultured in 96-well plates at a density of 1,000 cells/well and treated with different concentrations of **5d** (0.25-10 µM) for 72 h. Cells were incubated with detection reagent (100 µL) for 60 min at 37 °C with 5% CO_2. The fluorescence of each well was read at 485/535nm by plate reader (BioTek).

The results indicated that **5d** inhibited the growth of several types of NSCLC cell in a dose-dependent manner (see Figure 1). The inhibitory effect became visible at 1 µM and reached about 90% inhibition of cell proliferation at a concentration of 10 µM on A549 cells. **5d** also inhibited the proliferation of H1650, H1299 and HCC827 cells about 80-90% at 10 µM. Taken together, these results demonstrate that **5d** inhibits the proliferation of several types of NSCLC cells.

Clonogenic assay. Cells were seeded in 12-well plates (1000 cells per well in 2 mL of RPMI medium (#61970-010, Gibco) containing 10% (v/v) fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin (#10378016, Gibco)) and incubated overnight. The cells were treated with corresponding inhibitors for 5 days. Subsequently, the medium was carefully removed, and cells were fixed with 4% (v/v) paraformaldehyde for 20 min and stained with 0.5% (w/v) crystal violet for 20 min. After washing, the image of each well was photographed. To quantify the staining, 10% acetic acid was utilized to dissolve the colonies. The absorbance at a wavelength of 590 nm was measured to represent the relative cell number by comparing with the DMSO-treated group.

The results of colony formation assays (see Figure 2) showed that **5d** potently inhibited cell growth of the four NSCLC cell lines that were tested. A dose-dependent reduction in both colony number and size in **5d**-treated cells as compared with controls was observed. The amount of colonies in each well was quantified by the mass of attached crystal violet. **5d** suppresses around 90% of colony formation of all the four NSCLC cells at 10 µM. Notably, 5 µM **5d** inhibits the growth of A549 and HCC827 cells by 70% and 39%, respectively. These results confirm the efficacy of **5d** on inhibition of NSCLC cells proliferation.

Tumor spheroid assay. A 3D spheroid model was employed to investigate the effect of longer-term 5d treatment in a more complex model of tumor growth. The 3D spheroid model was established using A549 cancer cells by a method adapted from Feng et al. (*J. Med. Chem.* **2015**, *58*, 6456–6480). Each spheroid was prepared from 1000 A549 cells. The A549 cells (1000 cells/well) were seeded onto a 96-well round-bottomed ultra low attachment plate (Corning). After 2 days of incubation without disturbance, the spheroid was treated with indicated compound every 3 days. Images were captured and the diameter of each tumor spheroid were measured on the indicated days post-treatment using an inverted microscope (Nikon Eclipse Ti) connected with a NIS-elements software. The data were analyzed and plotted with Graphpad Prism8.

After five-day culturing, these spheroids were treated with 1, 2, or 5 μM of **5d** with 72 hours intervals over 12 days. Spheroid growth was monitored by measuring the diameter and this was compared to day 0 of the treatment. The tumor spheroids treated with **5d** were significant smaller compared to the control group (see Figure 3). With continuous exposure to 1, 2, or 5 μM of **5d** for 12 days, the growth of the spheroid tumor volume was inhibited by 40%, 63%, and 79%, respectively. These results indicate that the MIF2 tautomerase inhibitor **5d** effectively inhibits proliferation of A549 cancer cells in a spheroid tumor model.

Flow cytometry. For cell cycle analysis, A549 cells were seeded in 6-well plates at a density of 1×10^5 per well. The next day, the cells were treated with **5d** or vehicle for 48h. Subsequently, the cells were washed with PBS (3×) and then harvested after trypinzation. After centrifugation at 300g, the cells were incubated with a solution containing 20 μg/mL propidium iodide (PI) (Sigma, P4864) and 0.1% (v/v) Triton-X100 (Sigma, T8787) for 15 min at room temperature. Fluorescence was detected by a Cytoflex flow cytometer (Beckman Coulter, Woerden, the Netherlands) immediately. 30,000 cells were collected for each sample. Data were analyzed using FlowJo software (Tree start, Ashland, USA).

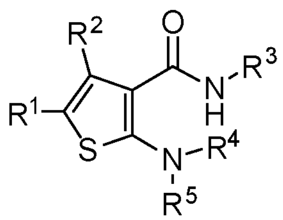
5d arrests A549 cells at G0/G1 phase of the cell cycle. The effect of **5d** on cell cycle progression was analyzed using flow cytometry. A549 cells were treated with different concentration of **5d** for a duration of 48 hours before analysis. The results showed that **5d** dose-dependently induced cell cycle arrest at the G0/G1 phase (see Figure 4). The percentage of A549 cells in G0/G1 phases was 56% for the control group. This percentage increased to 58%, 63%, and 67% upon treatment with 5, 7.5, and 10 μM **5d**, respectively. This result indicate **5d** induces inhibition of cell cycle progression, which can explain the observed inhibition of cell proliferation.

ERK signaling pathway study. A549 cells (3×10^5 cells per well) were seeded into each well of a 6-well plate with 2 mL RPMI-1640 medium containing 0.5% FBS (Costar Europe, Badhoevedorp, The Netherlands), and 1% penicillin/streptomycin solution (Corning). After overnight culturing, the cells were stimulated with MIF2 (100ng/mL in FBS free medium) or a mixture of MIF2 and different concentration of **5d** for 15 minutes. After that, cells were lysed by RIPA buffer containing 1× PhosSTOP and protease inhibitor (PI) cocktail (Roche, Mannheim, Germany). The BCA Protein Assay Kit (Pierce, Rockford IL, USA) was used to determine the protein concentration. 20 µg protein was separated by a pre-cast 10% NuPAGE Bis-Tris gel (Invitrogen, USA) and then transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% of skimmed milk for 1 hour at room temperature and incubation with the appropriate primary antibody (pERK, #9101, Cell Signaling, 1:1000; GAPDH, #97166, Cell Signaling, 1:10000) overnight at 4 °C, the membrane was treated with an HRP-conjugated secondary goat anti-rabbit antibody (#P0448, Dako, 1:2000) or rabbit anti-mouse antibody (#P0260, Dako, 1:2000) at room temperature for 1 hour. The protein bands were visualized with enhanced chemiluminescence (ECL) solution (GE Healthcare). The figures were quantified with imageJ software based on grayscale.

5d inhibits ERK signaling. The effect of treatment with **5d** on MIF2-related signaling pathways was investigated by assessment of MIF2-induced ERK phosphorylation using western blot analysis. Towards this aim, A549 cells were stimulated with MIF2 or **5d** pre-incubated MIF2 for 15 minutes, subsequently ERK phosphorylation was detected using western blot. MIF2 stimulated the ERK phosphorylation level of A549 cells to about 4.5 folds of control. This stimulation was attenuated by **5d** in a dose-dependent manner (see Figure 5). After pre-incubation with 10 µM **5d**, MIF2 only activated the ERK phosphorylation level to two folds of control. These data demonstrate that **5d** treatment inhibits ERK phosphorylation as a MIF2-related signaling event.

The invention furthermore relates to the following clauses:

Clause 1. Compound of structure (I)



(I)

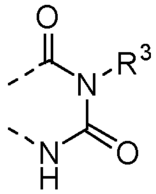
or a pharmaceutically acceptable salt thereof, wherein

R^1 represents H or optionally substituted phenyl;

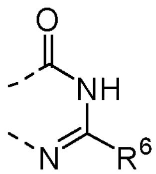
R^2 represents H, straight or branched C_{1-8} alkyl, straight or branched C_{2-8} alkenyl, or optionally substituted phenyl;

R^3 represents straight or branched C_{1-8} alkyl, straight or branched C_{2-8} alkenyl, phenyl, benzyl, phenethyl, naphthyl, naphthalen-1-ylmethyl, naphthalen-2-ylmethyl, naphthalen-1-yl-2-ethyl, or naphthalen-2-yl-2-ethyl, all optionally substituted with one or more Cl, F, Br, CH_3, C_2H_5, CCl_3, CF_3 or CBr_3 groups;

R^5 represents H, and R^4 represents H or C_{1-8} alkanoyl; or
R^3, the amide group to which R^3 is attached, R^4, R^5, and the nitrogen atom to which R^4 and R^5 are attached together form a bivalent radical with structure

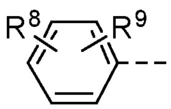


wherein R^3 is as defined above; or
R^3, the amide group to which R^3 is attached, R^4, R^5, and the nitrogen atom to which R^4 and R^5 are attached together form a bivalent radical with structure



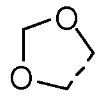
wherein R^6 represents H, Cl, F, Br, or
wherein R^6 represents straight or branched C^{1-8} alkyl, straight or branched C^{2-8} alkenyl, phenyl, benzyl, phenethyl, naphthyl, naphthalen-1-ylmethyl, naphthalen-2-ylmethyl, naphthalen-1-yl-2-ethyl, or naphthalen-2-yl-2-ethyl, all optionally substituted with one or more Cl, F, Br, CH^3, C^2H^5, CCl^3, CF^3 or CBr^3 groups.

Clause 2. Compound or pharmaceutically acceptable salt thereof according to clause 1, wherein
R^1 represents



,

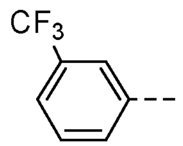
wherein R^8 and R^9 each independently represent H, F, Cl, Br, straight or branched C_{1-4} alkyl, CCl_3, CBr_3, or CF_3, or wherein R^8 and R^9 are attached to adjacent carbon atoms and together

with these adjacent carbon atoms form a five- or six-membered homo- or heterocyclic ring, for example a dioxolane ring with structure  .

Clause 3. Compound or pharmaceutically acceptable salt thereof according to clause 2, wherein at least one of R^8 and R^9 is F, Cl, Br, CCl_3, CBr_3, or CF_3.

Clause 4. Compound or pharmaceutically acceptable salt thereof according to clause 2, wherein both R^8 and R^9 are F, Cl or Br.

Clause 5. Compound or pharmaceutically acceptable salt thereof according to any one of the preceding clauses, wherein R^1 represents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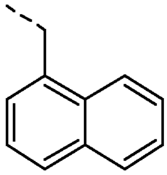
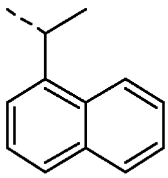
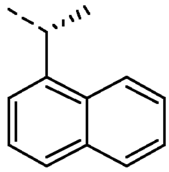
Clause 6. Compound or pharmaceutically acceptable salt thereof according to any one of the preceding clauses, wherein R^2 represents H, straight or branched C_{1-4} alkyl or straight or branched C_{2-4} alkenyl, most preferably methyl.

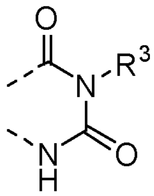
Clause 7. Compound or pharmaceutically acceptable salt thereof according to any one of the preceding clauses, wherein if R^2 is unsubstituted phenyl, then R^3 is not unsubstituted phenyl, and vice versa.

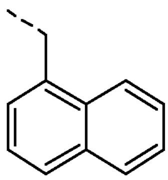
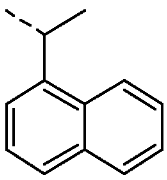
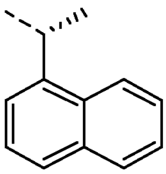
Clause 8. Compound or pharmaceutically acceptable salt thereof according to any one of the preceding clauses, wherein R^3 represents benzyl, phenethyl, naphthyl, naphthalen-1-ylmethyl, naphthalen-2-ylmethyl, naphthalen-1-yl-2-ethyl, or naphthalen-2-yl-2-ethyl, all optionally substituted with one or more Cl, F, Br, CH_3, C_2H_5, CCl_3, CF_3 or CBr_3 groups.

Clause 9. Compound or pharmaceutically acceptable salt thereof according to any one of the preceding clauses, wherein R^3 represents naphthalen-1-ylmethyl  or naphthalen-1-yl-2-ethyl , most preferably .

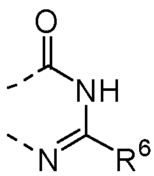
Clause 10. Compound or pharmaceutically acceptable salt thereof according to any one of the preceding clauses, wherein R^4 and R^5 represent H.

Clause 11. Compound or pharmaceutically acceptable salt thereof according to any one of clauses 1 – 9, wherein the amide group to which R^3 is attached, R^4, R^5, and the nitrogen atom to which R^4 and R^5 are attached together form a bivalent radical with structure



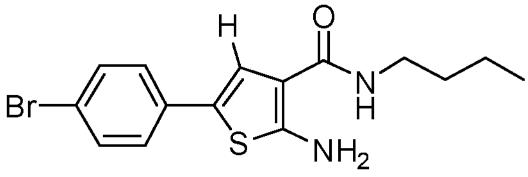
wherein R^3 represents naphthalen-1-ylmethyl  or naphthalen-1-yl-2-ethyl , preferably .

Clause 12. Compound or pharmaceutically acceptable salt thereof according to any one of clauses 1 – 9, wherein R^3, the amide group to which R^3 is attached, R^4, R^5, and the nitrogen atom to which R^4 and R^5 are attached together form a bivalent radical with structure



wherein R^6 represents Cl, F, Br, or phenyl optionally substituted with one or more Cl, F, Br, C_{1-4} alkyl, C_{1-4} alkoxy,, CCl_3, CF_3 or CBr_3 groups.

Clause 13. Compound, which is:



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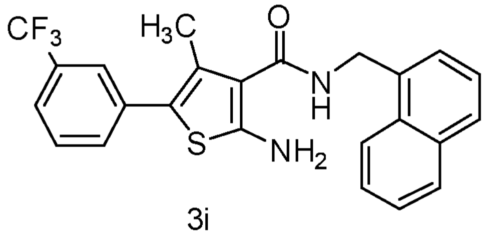

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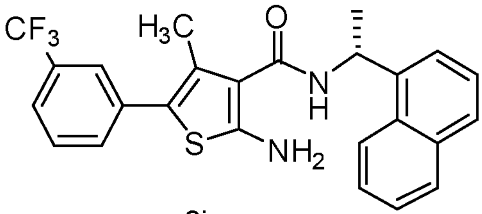

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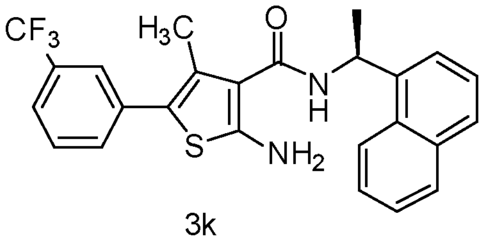

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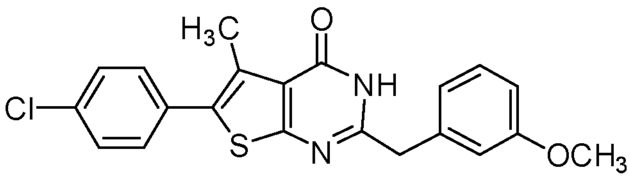

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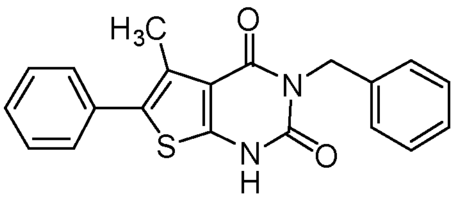

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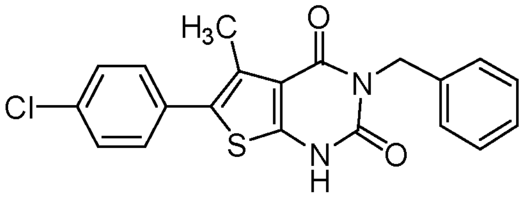

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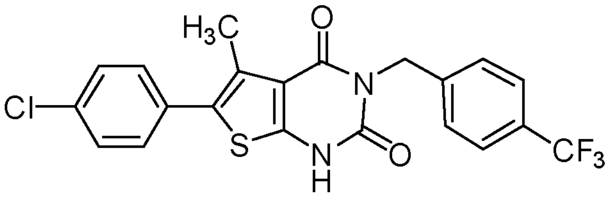

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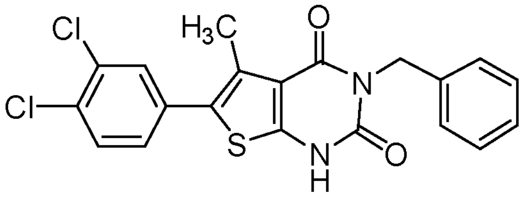

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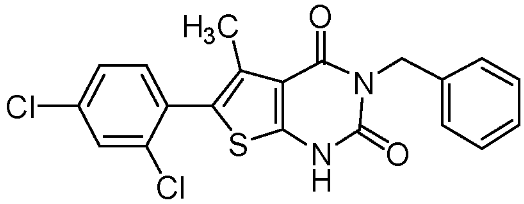

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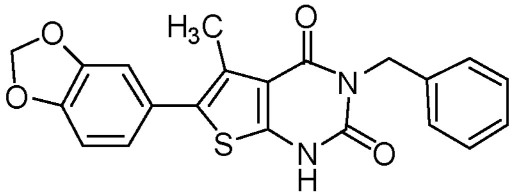

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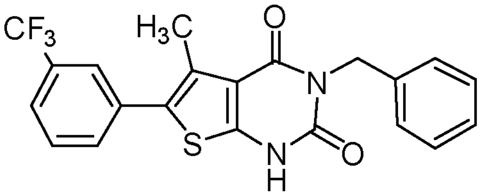

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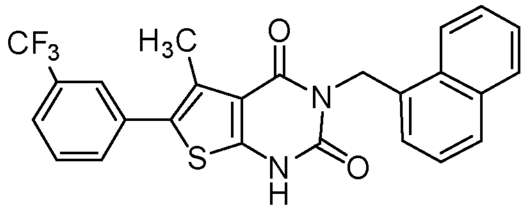

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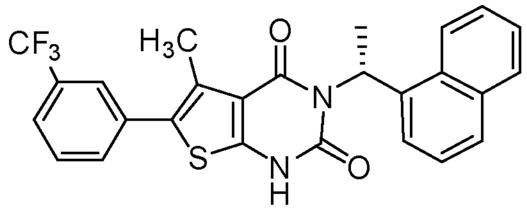

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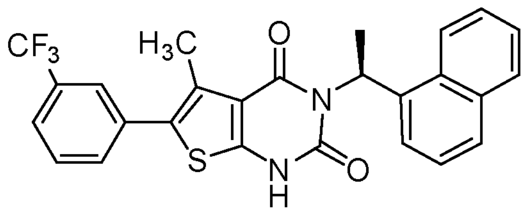
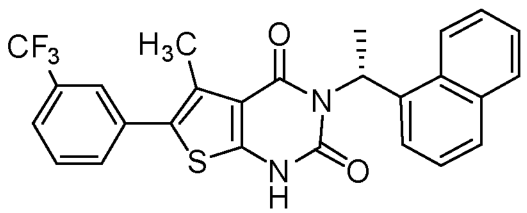
, or



5e

, or a pharmaceutically acceptable salt thereof.

Clause 14. Compound according to clause 13, which is



5d

(*(R)-5-Methyl-3-(1-(naphthalen-1-yl)ethyl)-6-(3-(trifluoromethyl)phenyl)thieno[2,3-d]pyrimidine-2,4(1H,3H)-dione)*, or a pharmaceutically acceptable salt thereof.

Clause 15. Pharmaceutical composition comprising a compound according to any one of the preceding clauses or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable adjuvant, carrier or vehicle.

Clause 16. Pharmaceutical composition according to clause 15, further comprising a macrophage migration inhibitory factor (MIF) inhibitor.

Clause 17. Compound or pharmaceutically acceptable salt thereof according to any one of clauses 1 – 14, or a pharmaceutical composition according to clause 15 or 16 for use as a medicament.

Clause 18. Compound or pharmaceutically acceptable salt thereof according to any one of clauses 1 – 14 or a pharmaceutical composition according to clause 15 or 16 for use in treating cancer or inflammatory disease, preferably for use in treating cancer, more preferably for use in treating a cancer chosen from the group consisting of melanoma, non-small-cell lung carcinoma (NSCLC), colorectal cancer, ovarian cancer, thyroid cancer, hairy cell leukemia, prostate cancer, glioblastoma, breast cancer, oral cancer, neuroblastoma, renal tumors, pancreatic cancer and genitourinary cancer, more preferably for use in treating NSCLC.

Clause 19. Compound or pharmaceutically acceptable salt thereof according to any one of clauses 1 – 14 or a pharmaceutical composition according to clause 15 or 16 for use as medicament, wherein the use is as a MIF2 inhibitor.

Clause 20. Compound or pharmaceutically acceptable salt thereof according to any one of clauses 1 – 14 or a pharmaceutical composition according to clause 15 or 16 for use in treating a disorder in which DDT tautomerase inhibition relieves the pathological process, preferably a disorder in which activation of the MAP/ERK and/or the PI3K/Akt pathway results in uncontrolled cell proliferation, more preferably a disorder which is characterized by overexpression of MIF2.

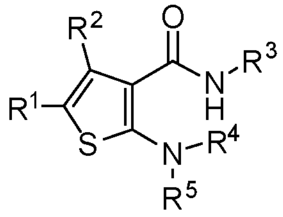
Clause 21. Method for treating cancer or inflammatory disease in a patient in need thereof, comprising administering to said patient an effective amount of the compound or pharmaceutically acceptable salt thereof of any one of clauses 1 – 14 or the pharmaceutical composition according to clause 15 or 16.

Clause 22. Method for treating NSCLC in a patient in need thereof, comprising administering to said patient an effective amount of the compound or pharmaceutically acceptable salt thereof of any one of clauses 1 – 14 or the pharmaceutical composition according to clause 15 or 16.

Clause 23. Method for treating a disorder characterized by overexpression of MIF2 in a patient in need thereof, comprising administering to said patient an effective amount of the compound or pharmaceutically acceptable salt thereof of any one of clauses 1 – 14 or the pharmaceutical composition according to clause 15 or 16.

CLAIMS

1. Compound of structure (I)



(I)

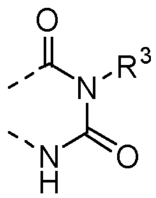
or a pharmaceutically acceptable salt thereof, wherein

R^1 represents H or optionally substituted phenyl;

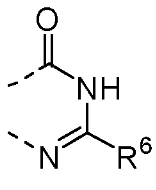
R^2 represents H, straight or branched C_{1-8} alkyl, straight or branched C_{2-8} alkenyl, or optionally substituted phenyl;

R^3 represents straight or branched C_{1-8} alkyl, straight or branched C_{2-8} alkenyl, phenyl, benzyl, phenethyl, naphthyl, naphthalen-1-ylmethyl, naphthalen-2-ylmethyl, naphthalen-1-yl-2-ethyl, or naphthalen-2-yl-2-ethyl, all optionally substituted with one or more Cl, F, Br, CH_3, C_2H_5, CCl_3, CF_3 or CBr_3 groups;

R^5 represents H, and R^4 represents H or C_{1-8} alkanoyl; or
R^3, the amide group to which R^3 is attached, R^4, R^5, and the nitrogen atom to which R^4 and R^5 are attached together form a bivalent radical with structure



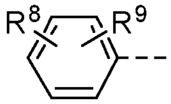
wherein R^3 is as defined above; or
R^3, the amide group to which R^3 is attached, R^4, R^5, and the nitrogen atom to which R^4 and R^5 are attached together form a bivalent radical with structure



wherein R^6 represents H, Cl, F, Br, or
wherein R^6 represents straight or branched C^{1-8} alkyl, straight or branched C^{2-8} alkenyl, phenyl, benzyl, phenethyl, naphthyl, naphthalen-1-ylmethyl, naphthalen-2-ylmethyl,

naphthalen-1-yl-2-ethyl, or naphthalen-2-yl-2-ethyl, all optionally substituted with one or more Cl, F, Br, CH^3, C^2H^5, CCl^3, CF^3 or CBr^3 groups.

2. Compound or pharmaceutically acceptable salt thereof according to claim 1, wherein R^1 represents



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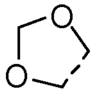
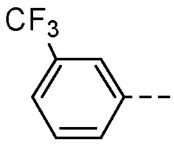
wherein R^8 and R^9 each independently represent H, F, Cl, Br, straight or branched C_{1-4} alkyl, CCl_3, CBr_3, or CF_3, or wherein R^8 and R^9 are attached to adjacent carbon atoms and together with these adjacent carbon atoms form a five- or six-membered homo- or heterocyclic ring, for example a dioxolane ring with structure  .

3. Compound or pharmaceutically acceptable salt thereof according to claim 2, wherein at least one of R^8 and R^9 is F, Cl, Br, CCl_3, CBr_3, or CF_3.

4. Compound or pharmaceutically acceptable salt thereof according to claim 2, wherein both R^8 and R^9 are F, Cl or Br.

5. Compound or pharmaceutically acceptable salt thereof according to claim 3, wherein R^1 represents



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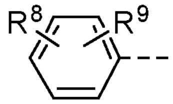
6. Compound or pharmaceutically acceptable salt thereof according to any one of the preceding claims, wherein R^2 represents H, straight or branched C_{1-4} alkyl or straight or branched C_{2-4} alkenyl, preferably wherein R^2 represents straight or branched C_{1-4} alkyl or straight or branched C_{2-4} alkenyl, most preferably methyl.

7. Compound or pharmaceutically acceptable salt thereof according to any one of the preceding claims, wherein R^3 represents benzyl, phenethyl, naphthyl, naphthalen-1-ylmethyl, naphthalen-2-ylmethyl, naphthalen-1-yl-2-ethyl, or naphthalen-2-yl-2-ethyl, all optionally substituted with one or more Cl, F, Br, CH_3, C_2H_5, CCl_3, CF_3 or CBr_3 groups.

8. Compound or pharmaceutically acceptable salt thereof according to any one of the preceding claims, wherein

R^1 represents



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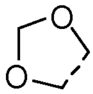
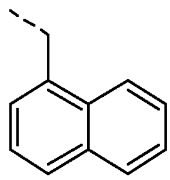
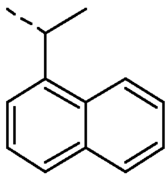
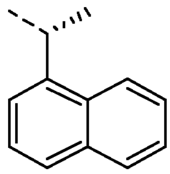
wherein R^8 and R^9 each independently represent H, F, Cl, Br, straight or branched C_{1-4} alkyl, CCl_3, CBr_3, or CF_3, or wherein R^8 and R^9 are attached to adjacent carbon atoms and together with these adjacent carbon atoms form a five- or six-membered homo- or heterocyclic ring, for example a dioxolane ring with structure  , and wherein

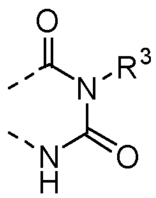
R^2 represents straight or branched C_{1-4} alkyl or straight or branched C_{2-4} alkenyl, most preferably methyl, and wherein

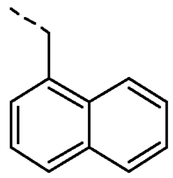
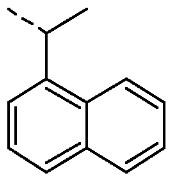
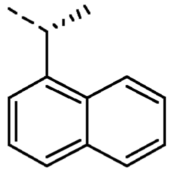
R^3 represents benzyl, phenethyl, naphthyl, naphthalen-1-ylmethyl, naphthalen-2-ylmethyl, naphthalen-1-yl-2-ethyl, or naphthalen-2-yl-2-ethyl, all optionally substituted with one or more Cl, F, Br, CH_3, C_2H_5, CCl_3, CF_3 or CBr_3 groups.

9. Compound or pharmaceutically acceptable salt thereof according to any one of the preceding claims, wherein R^3 represents naphthalen-1-ylmethyl  or naphthalen-1-yl-2-ethyl  , most preferably  .

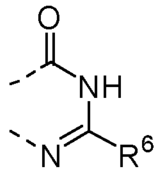
10. Compound or pharmaceutically acceptable salt thereof according to any one of the preceding claims, wherein R^4 and R^5 represent H.

11. Compound or pharmaceutically acceptable salt thereof according to any one of claims 1 – 9, wherein the amide group to which R^3 is attached, R^4, R^5, and the nitrogen atom to which R^4 and R^5 are attached together form a bivalent radical with structure



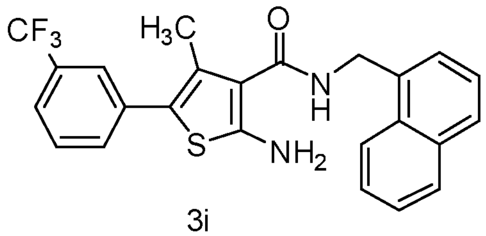
wherein R^3 represents naphthalen-1-ylmethyl  or naphthalen-1-yl-2-ethyl , preferably .

12. Compound or pharmaceutically acceptable salt thereof according to any one of claims 1 – 9, wherein R^3, the amide group to which R^3 is attached, R^4, R^5, and the nitrogen atom to which R^4 and R^5 are attached together form a bivalent radical with structure



wherein R^6 represents Cl, F, Br, or phenyl optionally substituted with one or more Cl, F, Br, C_{1-4} alkyl, C_{1-4} alkoxy,, CCl_3, CF_3 or CBr_3 groups.

13. Compound, which is:



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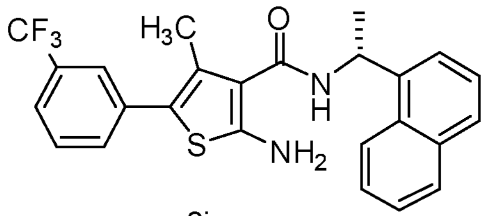

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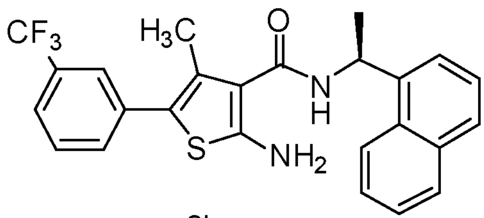

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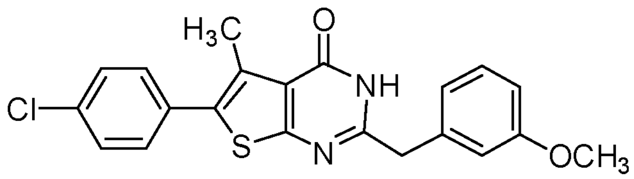

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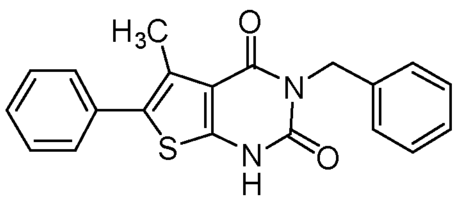

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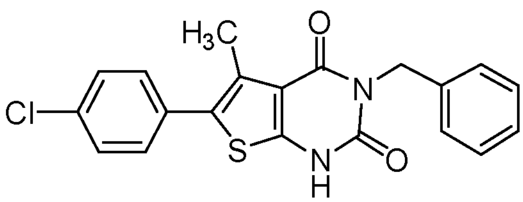

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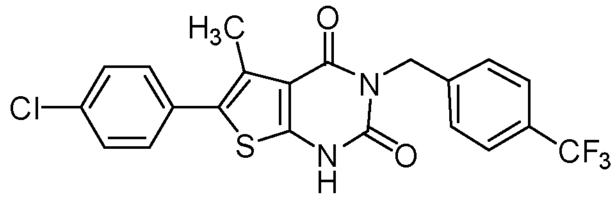

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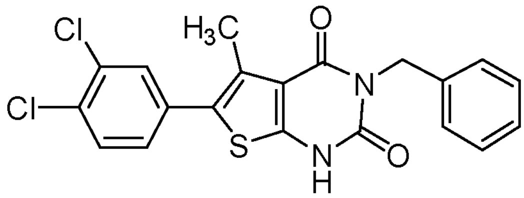

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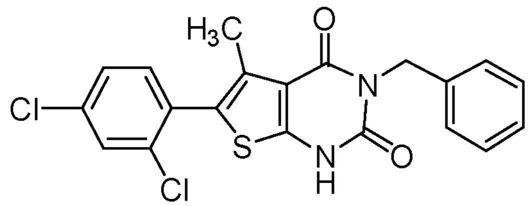

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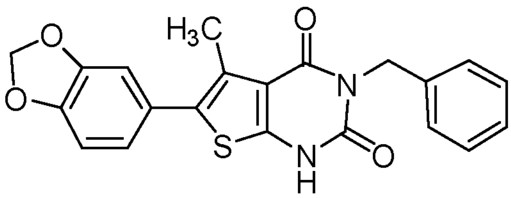

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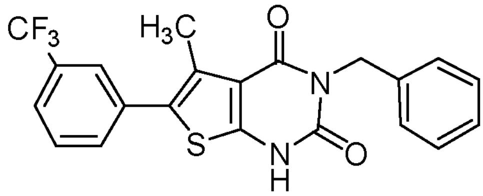

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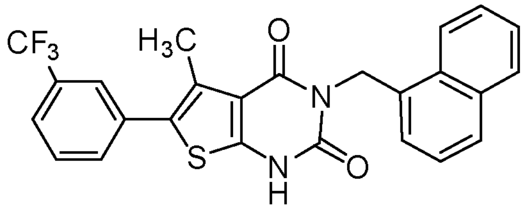

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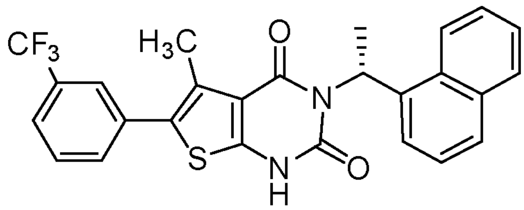
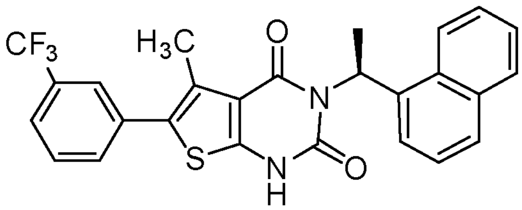
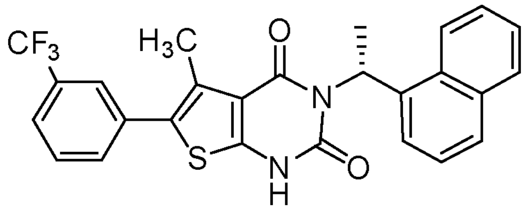


5d , or



5e , or a pharmaceutically acceptable salt thereof.

14. Compound according to claim 13, which is



5d

(*(R)-5-Methyl-3-(1-(naphthalen-1-yl)ethyl)-6-(3-(trifluoromethyl)phenyl)thieno[2,3-d]pyrimidine-2,4(1H,3H)-dione)*, or a pharmaceutically acceptable salt thereof.

15. Pharmaceutical composition comprising a compound according to any one of the preceding claims or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable adjuvant, carrier or vehicle.

16. Pharmaceutical composition according to claim 15, further comprising a macrophage migration inhibitory factor (MIF) inhibitor.

17. Compound or pharmaceutically acceptable salt thereof according to any one of claims 1 – 14, or a pharmaceutical composition according to claim 15 or 16 for use as a medicament.

18. Compound or pharmaceutically acceptable salt thereof according to any one of claims 1 – 14 or a pharmaceutical composition according to claim 15 or 16 for use in treating cancer or inflammatory disease, preferably for use in treating cancer, more preferably for use in treating a cancer chosen from the group consisting of melanoma, acute myeloid leukemia (AML), non-small-cell lung carcinoma (NSCLC), colorectal cancer, ovarian cancer, thyroid cancer, hairy cell leukemia, prostate cancer, glioblastoma, breast cancer, oral cancer, neuroblastoma, renal tumors, pancreatic cancer and genitourinary cancer, more preferably for use in treating AML or NSCLC, most preferably for use in treating NSCLC.

19. Compound or pharmaceutically acceptable salt thereof according to any one of claims 1 – 14 or a pharmaceutical composition according to claim 15 or 16 for use as medicament, wherein the use is as a MIF2 inhibitor.

20. Compound or pharmaceutically acceptable salt thereof according to any one of claims 1 – 14 or a pharmaceutical composition according to claim 15 or 16 for use in treating a disorder in which MIF2 inhibition relieves the pathological process, preferably a disorder in which activation of the MAP/ERK and/or the PI3K/Akt pathway results in uncontrolled cell proliferation, more preferably a disorder which is characterized by overexpression of MIF2.

21. Method for treating cancer or inflammatory disease in a patient in need thereof, comprising administering to said patient an effective amount of the compound or pharmaceutically acceptable salt thereof of any one of claims 1 – 14 or the pharmaceutical composition according to claim 15 or 16.

22. Method for treating NSCLC in a patient in need thereof, comprising administering to said patient an effective amount of the compound or pharmaceutically acceptable salt thereof of any one of claims 1 – 14 or the pharmaceutical composition according to claim 15 or 16.

23. Method for treating a disorder characterized by overexpression of MIF2 in a patient in need thereof, comprising administering to said patient an effective amount of the compound

or pharmaceutically acceptable salt thereof of any one of claims 1 – 14 or the pharmaceutical composition according to claim 15 or 16.

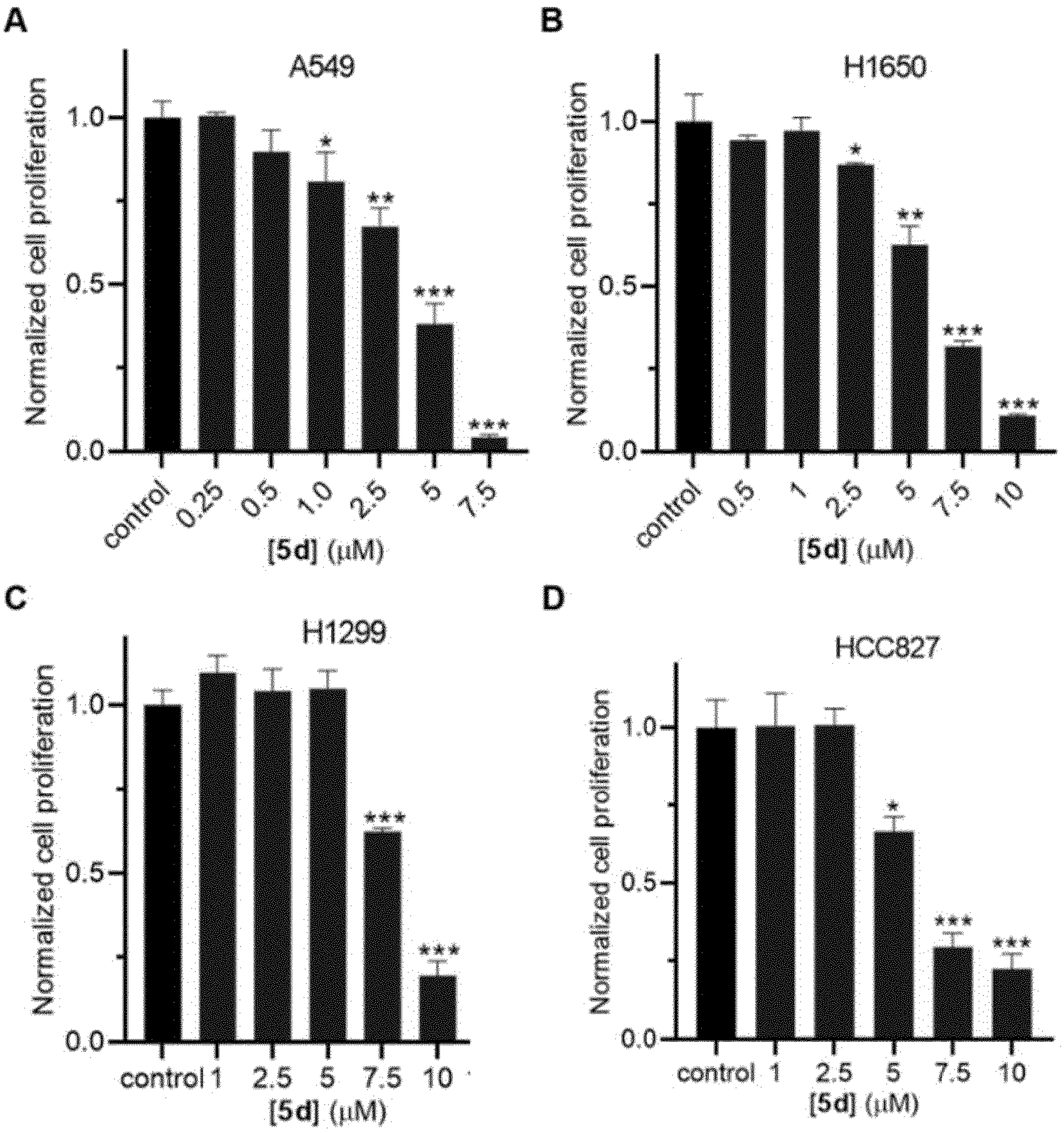

A
A549
Normalized cell proliferation
1.0
0.5
0.0
*
**
***
***
control
0.25
0.5
1.0
2.5
5
7.5
[5d] (μM)
B
H1650
Normalized cell proliferation
1.0
0.5
0.0
*
**
***
***
control
0.5
1
2.5
5
7.5
10
[5d] (μM)
C
H1299
Normalized cell proliferation
1.0
0.5
0.0
***
***
control
1
2.5
5
7.5
10
[5d] (μM)
D
HCC827
Normalized cell proliferation
1.0
0.5
0.0
*
***
***
control
1
2.5
5
7.5
10
[5d] (μM)

FIG.1

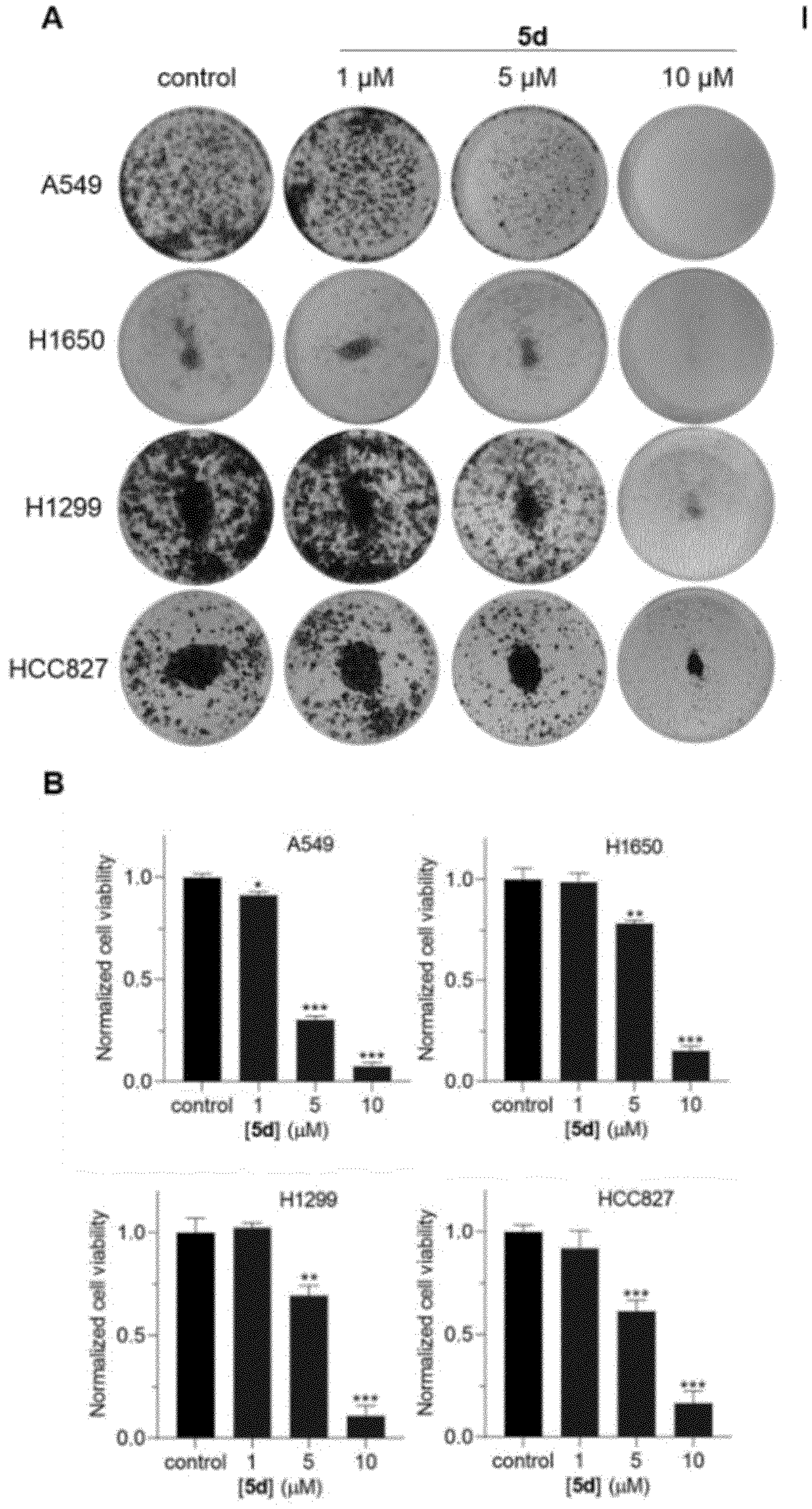

A
5d
control
1 μM
5 μM
10 μM
A549
H1650
H1299
HCC827
B
A549
H1650
H1299
HCC827
Normalized cell viability
1.0
0.5
0.0
control
1
5
10
[5d] (μM)

FIG. 2

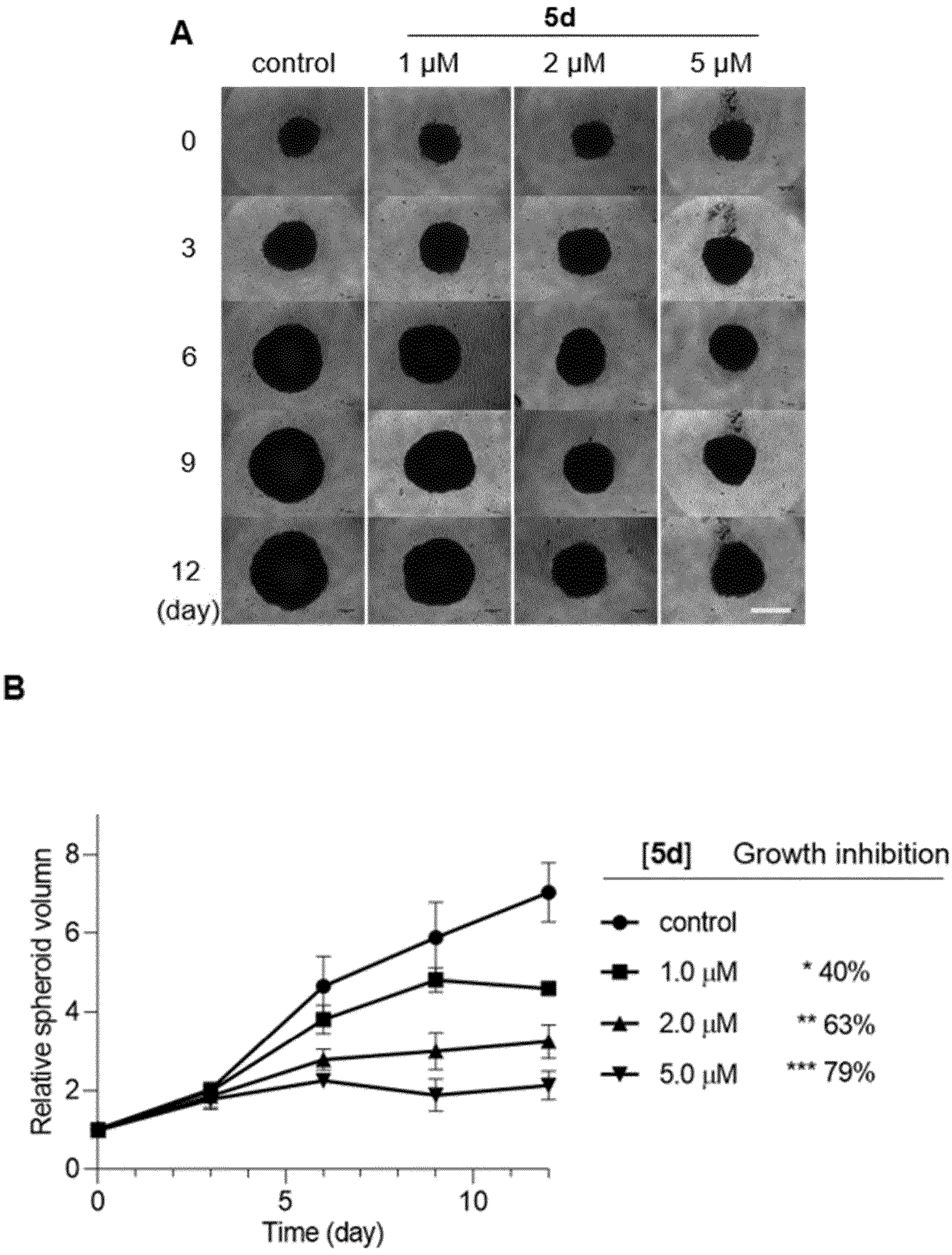
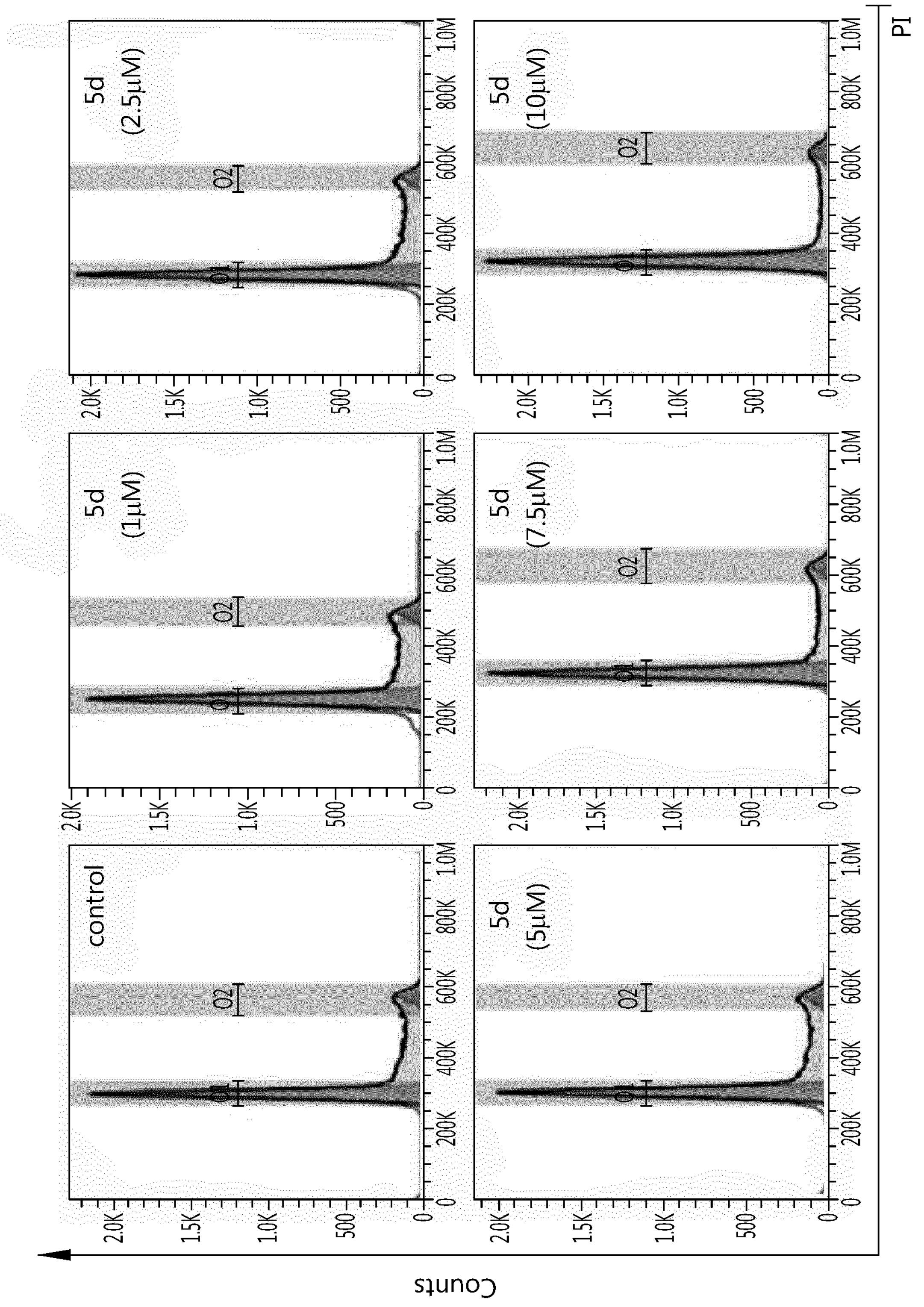

A
5d
control
1 μM
2 μM
5 μM
0
3
6
9
12
(day)
B
Relative spheroid volumn
0
2
4
6
8
0
5
10
Time (day)
[5d]
Growth inhibition
control
1.0 μM
* 40%
2.0 μM
** 63%
5.0 μM
*** 79%

FIG. 3

Fig. 4A



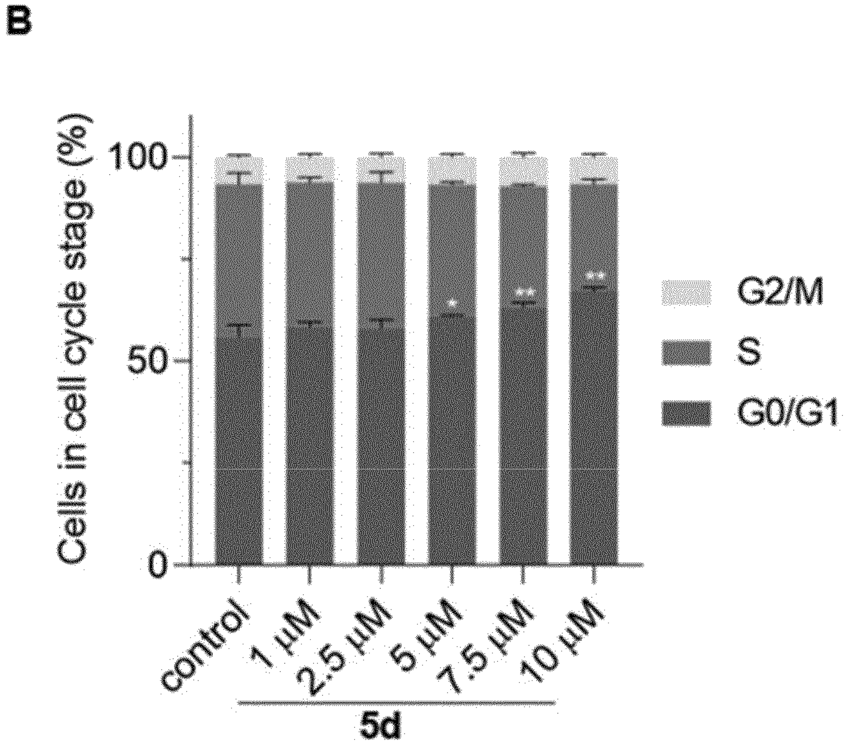

B
Cells in cell cycle stage (%)
100
50
0
G2/M
S
G0/G1
*
**
**
control
1 µM
2.5 µM
5 µM
7.5 µM
10 µM
5d

FIG. 4

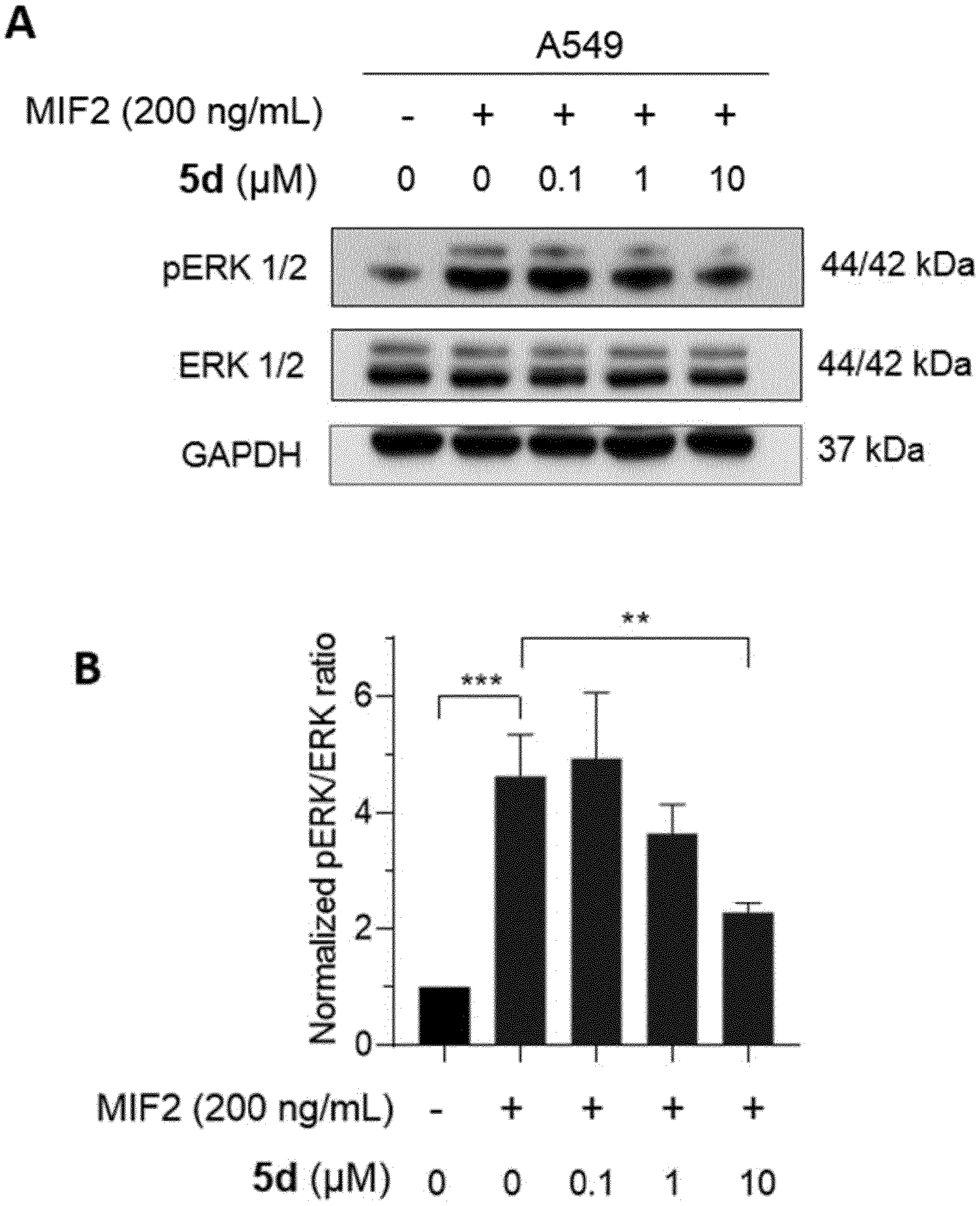

A
A549
MIF2 (200 ng/mL) - + + + +
5d (μM) 0 0 0.1 1 10
pERK 1/2
44/42 kDa
ERK 1/2
44/42 kDa
GAPDH
37 kDa
B
Normalized pERK/ERK ratio
0
2
4
6
***
**
MIF2 (200 ng/mL) - + + + +
5d (μM) 0 0 0.1 1 10

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2022/074153

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07D333/38 C07D495/04 A61K31/381 A61K31/519 A61P35/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07D A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	N. ELEFTHERIADIS ET. AL.: "Design of a Novel Thiophene Inhibitor of 15-Lipoxygenase-1 with Both Anti-inflammatory and Neuroprotective Properties", EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY, vol. 122, 21 October 2016 (2016-10-21), pages 786-801, XP029705965, DOI: 10.1016/j.ejmech.2016.07.101 Fgure 2, Scheme 1, Tables -----	1-3,6, 10,15,17
	-/--	

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search	Date of mailing of the international search report
4 November 2022	11/11/2022

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Helps, Ian

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	W.WANG ET.AL.: "Design, Synthesis and Biological Evaluation of Novel 3,4,5-Trisubstituted Aminothiophenes as Inhibitors of p53-MDM2 Interaction. Part 1.", BIOORGANIC AND MEDICINAL CHEMISTRY, vol. 21, no. 11, 1 June 2013 (2013-06-01), pages 2879-2885, XP028535202, DOI: 10.1016/j.bmc.2013.03.061 Scheme 1, Table 1, compounds 23-25 -----	1-3,7, 10,15, 17,18,21
X	W. WANG ET. AL.: "Design, Synthesis and Biological Evaluation of Novle 3,4,5-Trisubsituted Aminothiophenes as Inhibitors of p53-MDM2 Interaction. Part 2.", BIOORGANIC AND MEDICINAL CHEMISTRY, vol. 21, no. 11, 1 June 2013 (2013-06-01), pages 2886-2894, XP028535206, DOI: 10.1016/j.bmc.2013.03.070 Table 1, compounds 4d, 4k, 4o, 4t, 6, 7d -----	1-3,7, 10,15, 17,18,21
X	W.M. ELGAHER ET. AL.: "Discovery and Structure-Based Optimization of 2-Ureido-thiophene-3-carboxylic Acids as Dual Bacterial RNA Polymerase and Viral Transcriptase Inhibitors.", JOURNAL OF MEDICINAL CHEMISTRY, vol. 59, no. 15, 24 June 2016 (2016-06-24), pages 7212-7222, XP002806575, DOI: 10.1021/acs.jmedchem.6b00730 page 7219, Scheme 3, compound 42 -----	1-4,6,10
X	S.SASAKI ET. AL.: "Discovery of a Thieno[2,3-d]pyrimidin-2,4-dione Bearing a p-Methoxyphenyl Moiety at the 6-Position. A Highly Potent and Orally Bioavailable Non-Peptide Antagonist for the Human Luteinizing Hormone Releasing Hormone Receptor.", JOURNAL OF MEDICINAL CHEMISTRY, vol. 46, no. 1, 27 November 2002 (2002-11-27), pages 113-124, XP002967759, DOI: 10.1021/jm020180i page 115, Scheme 1, compounds 4a to 4l -----	1,6
X	K. WANG ET. AL.: "Cyanoacetamide MCR (III) Three Component Gewald Reaction Revisited", JOURNAL OF COMBINATORIAL CHEMISTRY, vol. 12, 1 January 2010 (2010-01-01), pages 111-118, XP055075595, DOI: 10.1021/cc9001586 table 1 -----	1,2,6,10
1	-/--	

International application No
PCT/EP2022/074153

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	S. THANNE ET. AL.: "Synthesis and Evaluation of New 2-Aminothiophenes Against Mycobacterium Tuberculosis", ORGANIC AND BIOMOLECULAR CHEMISTRY, vol. 14, no. 25, 25 May 2016 (2016-05-25), pages 6119-6133, XP055923264, DOI: 10.1039/c6ob00821f page 6122, compounds 59 to 63; Figure 3 -----	1,2,6, 10,15,17
X	WO 03/029241 A1 (SMITHKLINE BEECHAM CORPORATION) 10 April 2003 (2003-04-10) claims; examples 8, 9, 12, 26 -----	1-3,6,10
A	FR 2 858 323 A1 (L'OREAL) 4 February 2005 (2005-02-04) page 2, line 10 - line 23; claims; examples -----	1-23

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2022/074153

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03029241 A1	10-04-2003	NONE	
FR 2858323 A1	04-02-2005	NONE	