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Design, synthesis and biological evaluation of novel 3,4,5-trisubstituted aminothiophenes as inhibitors of p53–MDM2 interaction. Part 1

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ABSTRACT

A series of 3,4,5-trisubstituted aminothiophenes were designed, synthesized, and evaluated for their p53–MDM2 binding inhibitory potency and anti-proliferation activities against A549 and PC3 tumor cell lines. Fourteen compounds had appreciably improved MDM2 binding affinities than lead compound MCL0527 (**3**) and a few compounds showed comparable activities to that of Nutlin-3. Meanwhile, most of the 3,4,5-trisubstituted aminothiophenes displayed better or equivalent anti-proliferation activities against wild-type p53 cell line A549 compared to that of Nutlin-3. Over ten compounds exhibited desirable selective profiles of p53 status. Particularly, compounds **9**, **16** and **18** displayed 22-, 6- and 22-fold selectivity of p53 status, respectively, much better than that of Nutlin-3 (fourfold).

1. Introduction

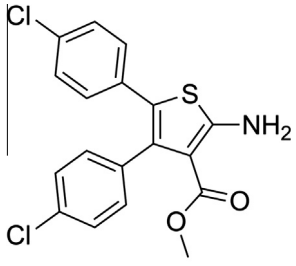
The tumor suppressor p53, key node of the p53 pathway, plays essential roles in various cellular activities, such as cell cycle, apoptosis and DNA repair.[1–4] It is an attractive molecule target for tumor treatment. However, p53 is one of the most frequently altered genes in human tumors. Approximately 50% of all human malignancies harbor mutations or deletions in the DNA-binding domain of p53.[5,6] Although the remaining 50% of all human tumors express wild-type p53, its activity can be inhibited by over-expression of MDM2 oncoprotein (murine double minute 2, also frequently referred to as HDM2 in human), the master negative regulator of p53.[7–9] Reactivation of p53 by utilizing small-molecule p53–MDM2 binding inhibitors is now recognized as a promising strategy for cancer therapy.[10–12]

In our previous work, a promising lead compound (MCL0527) with a novel scaffold was identified by a pharmacophore-based virtual screening strategy combining molecular docking studies (Fig. 1).[13] The biological characterization revealed that MCL0527 and its derivatives displayed good MDM2 binding affinities and anti-proliferative effects against four tumor cell lines. Herein, with the attempt to find better p53–MDM2 binding inhibitors, the structural modification and optimization of MCL0527 was carried out incorporating the following concepts: (1) retaining the 2-amino-4,5-bis(4-chlorophenyl)thiophene-3-carboxylic acid scaffold; (2) focusing on the modification at 3-carboxy group, using amides to replace esters; (3) derivatization at the 2-amino group to adjust the physicochemical properties. In this work, we reported the synthesis and biological evaluation of this new series of p53–MDM2 binding inhibitors. Besides, the preliminary structure–activity relationships (SARs) of the target compounds were discussed.

2. Results and discussion

2.1. Chemistry

The synthetic routes for 3,4,5-trisubstituted aminothiophene derivatives are summarized in Scheme 1. Reaction of 4-chloro-



MCL0527

Figure 1. Structure of the lead compound MCL0527.

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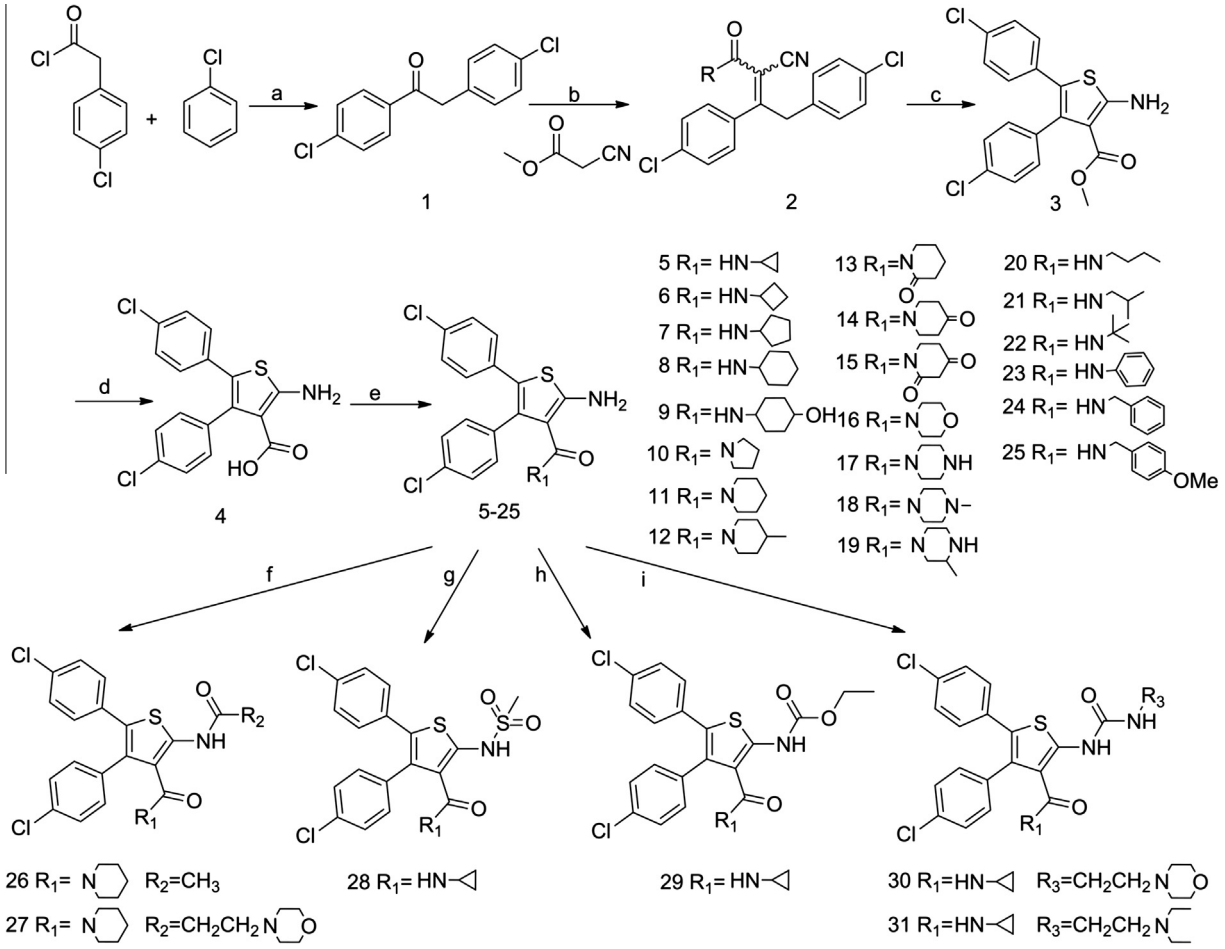


Scheme 1. Synthetic routes of target compounds (**5–31**). Reagents and conditions: (a) $AlCl_3$, CH_2Cl_2, rt; (b) methyl cyanoacetate, $TiCl_4$, pyridine, THF, rt; (c) sulfur, $NHEt_2$, THF, rt; (d) 2 N NaOH, EtOH, reflux; (e) EDCI, HOBt, amines, CH_2Cl_2, rt; (f) for **26**, acetyl chloride, TEA, CH_2Cl_2, rt; for **27**, chloropropionyl chloride, TEA, CH_2Cl_2, rt; morpholine, TEA, dioxane, reflux; (g) methylsulfonyl chloride, TEA, CH_2Cl_2, rt; (h) ethyl carbonochloridate, K_2CO_3, toluene, rt; (i) 2-chloroethyl isocyanate, TEA, CH_2Cl_2, reflux; TEA, dioxane, reflux, morpholine for **30**, diethylamine for **31**.

phenylacetylchloride with chlorobenzene gave the Friedel–Crafts acylation product **1**. According to the Gewald synthesis,[14] condensation of compound **1** with methyl cyanoacetate in the presence of $TiCl_4$ afforded the olefin intermediate **2**. Treatment of **2** with elemental sulfur and diethyl amine led to the lead compound **3** (MCL0527). Hydrolysis of **3** with 2 N NaOH aqueous solution produced the common intermediate **4**. Then, **4** was treated with appropriate amines in the presence of EDCI and HOBt yielded the desired compounds (**5**–**25**).

In addition, the 2-amino group in compounds **5** and **11** was converted into amides, sulfamides, carbamates and ureas, respectively. Acylation of **11** with acetyl chloride or chloropropionyl chloride and followed by reaction with morpholine obtained amides **26** and **27**. Sulfamide **28** was prepared by reaction of **5** with methylsulfonyl chloride. Treatment of **5** with ethyl chlorformate afforded the carbamate **29**. Reaction of **5** with 2-chloro ethyl isocyanate yielded chloroethylurea, which subsequently treated with corresponding secondary amines to give target compounds **30** and **31**.

2.2. p53–MDM2 binding inhibitory activities

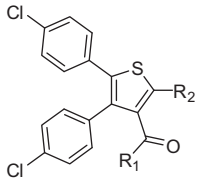
The obtained 3,4,5-trisubstituted aminothiophene compounds were evaluated for their p53–MDM2 binding inhibitory activities by fluorescence-polarization based binding assay (FP assay).[15,16] Nutlin-3 was used as positive control. The results are summarized in Table 1.

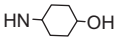
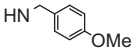
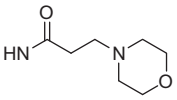
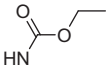
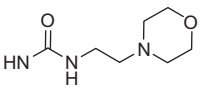
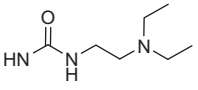
As shown in Table 1, most of the 3,4,5-trisubstituted aminothiophene derivatives exhibited micromolar to nanomolar affinities towards MDM2. Fourteen compounds showed improved binding inhibitory activities than that of MCL0527 (**3**) and a few compounds (**5**, **7**, **8**, **16**, **22** and **24**) displayed comparable activities to that of Nutlin-3. Most of the compounds with secondary carboxamides at the 3-position of thiophene ring (**5–9**, **20–22**, **24** and **25**) showed potent MDM2 binding affinities. Among them, *N*-benzyl carboxamide at the 3-position of thiophene ring (**24**, K_i = 0.15 μM) was the most preferred substituent for binding affinity. However, in the case of *N*-phenyl carboxamide (**23**), a significant decrease of potency was observed. On the other hand, compounds with aliphatic tertiary carboxamides at 3-position exhibited moderate to potent binding affinities (**10–19**). Comparing the binding affinities of **11**, **12** and **17–19** revealed that introducing heterocycles with two nitrogen-atoms at the end of carboxamide (**17–19**) caused a dramatic loss in potency. All 2-amino acylated derivatives (**26–31**) showed a remarkably lowered potency.

2.3. Tumor cell growth inhibition studies

All the synthesized compounds were further evaluated for their anti-proliferation activities against two human cancer cell lines, including one wild-type p53 cell line A549 and one p53 null cell line PC3. IC_{50} values were obtained using the standard SRB assay.

Table 1
P53–MDM2 binding inhibitory activities and tumor cell anti-proliferation activities of 3,4,5-trisubstituted aminothiophenes (**3** and **5–31**)



Compd	R1	R2	K_i[a] (μM)	SRB IC_{50} (μM)	
				A549[a]	PC3[a]
3 (MCL0527)[c]	OCH_3	NH_2	1.52	5.04	2.78
5[c]		NH_2	0.54	1.95	3.68
6		NH_2	1.12	12.48	24.79
7		NH_2	0.54	6.79	21.24
8[c]		NH_2	0.23	2.41	7.3
9		NH_2	0.99	0.25	5.67
10[c]		NH_2	0.74	0.89	6.22
11		NH_2	1.21	4.62	14.33
12		NH_2	0.80	1.75	5.75
13		NH_2	1.55	1.82	4.94
14		NH_2	2.19	2.01	1.34
15		NH_2	3.52	4.09	9.23
16		NH_2	0.43	1.87	11.69
17		NH_2	6.81	1.02	2.71
18		NH_2	1.52	0.59	13.14
19		NH_2	17.66	4.93	7.21
20		NH_2	0.55	8.17	17.75
21		NH_2	4.13	20.98	13.16
22		NH_2	0.81	8.85	15.14
23[c]		NH_2	>50	25.03	>50
24		NH_2	0.15	16.30	31.81
25		NH_2	0.64	18.23	29.54
26			3.57	6.01	10.32
27			NA[b]	>25	>25
28			>50	NT[d]	NT[d]
29			NA[b]	39.79	27.56
30			NA[b]	NT[d]	NT[d]
31			NA[b]	NT[d]	NT[d]
Nutlin-3	—	—	0.055	4.62	20.04

[a] Values are means of two experiments.
[b] NA, no activity.
[c] These compounds were reported in our previous studies.[13]
[d] NT, not tested.

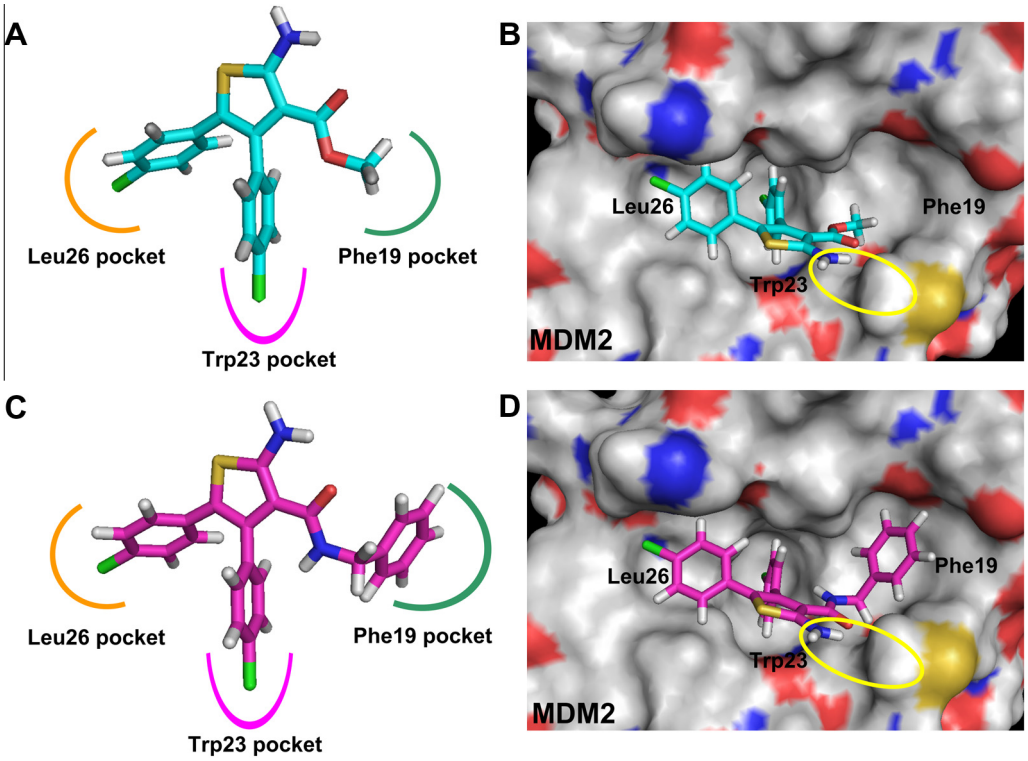


Figure 2. Molecular docking analysis of **3** and **24** with MDM2. (A) 3D schematic interaction model for **3** (cyan backbone) with MDM2. (B) Surface show of **3** (cyan backbone) bound to MDM2. (C) 3D schematic interaction model for **24** (pink backbone) with MDM2. (D) Surface show of **24** (pink backbone) bound to MDM2. Yellow circles indicated that the 2-amino group of thiophene ring was very close to the Met62 residue of MDM2.

According to the results (Table 1), several compounds (such as **7–13**, **16**, **18** and **22**) showed a good selectivity on wild-type p53 cell line A549 over p53 null cell line PC3. In particular, compounds **9**, **16** and **18** showed 22-, 6- and 22-fold selectivity of p53 status, respectively, much better than that of Nutlin-3 (fourfold). Besides, more than 10 compounds exhibited improved anti-proliferation activities against A549 cell line than that of Nutlin-3. Among them, compound **9** showed the best activity (IC_{50} = 0.25 μM). Unfortunately, although compound **24** was potent for MDM2 binding affinity, it did not exhibit satisfactory performance in tumor cell growth inhibition studies, possibly due to its poor solubility. Based on the above experimental results, it is noteworthy that compound **9** and **16** displayed potent activities not only in p53–MDM2 binding inhibition but also in tumor cell anti-proliferation, and had excellent selective profile on p53 status. Therefore, they can be used as promising lead compounds for further investigation.

2.4. Molecular docking studies

To examine possible binding modes of lead compound MCL0527 (**3**) and the most potent compound **24** with MDM2, a docking analysis utilizing the C-DOCKER program within Discovery Studio 2.1 software package was performed. The published X-ray crystal structure of MDM2 (PDB ID:1YCR) was used for the docking calculation. Similar binding modes of **3** and **24** with MDM2 were indicated in the docking results (Fig. 2). Herein, the thiophene ring, serving as an important core structure, played the role in projecting three hydrophobic substituents into the MDM2 binding cleft in proper directions. Two 4-chlorophenyl groups and one methyl ester/*N*-benzyl group mimiced the three key residues of p53 (Leu26, Trp23 and Phe19, respectively) to form the hydrophobic interactions with protein (Fig. 2A and C). For the lead compound **3** (Fig. 2B), a spare room not occupied by methyl ester at the Phe19 binding site was observed, and it could be perfectly satisfied by the *N*-benzyl group in compound **24** (Fig. 2D). In addition, the 2-amino group of thiophene ring was found very close to the Met62 residue in both molecule simulation results (highlighted by yellow circles, Fig. 2B and D). Derivatization at this position would possibly lead to direct contact between compounds and protein surface, which consequently influences the binding affinity. Thus, the results of molecular docking studies gave an explanation to the dramatic drop of potency observed in compounds with substituents at 2-amino group.

3. Conclusion

A series of novel 3,4,5-trisubstituted aminothiophene derivatives were designed and synthesized as p53–MDM2 binding inhibitors. In the MDM2 binding assay, most derivatives showed largely improved potency compared to lead compound MCL0527. Eleven compounds displayed potent binding affinities at nanomolar level, equivalent to that of Nutlin-3. In addition, more than ten compounds exhibited better anti-proliferation activities against A549 cell line than that of Nutlin-3. Several compounds showed at least 3-fold inhibitory selectivity on wild-type p53 cell line A549 over p53 null cell line PC3. This study validated the potential of developing 3,4,5-trisubstituted aminothiophene series as a tool for cancer therapy by targeting p53–MDM2 interaction. Further studies are still under investigation.

4. Experimental

4.1. Chemistry

Melting points were determined with a B-540 Büchi apparatus and are uncorrected. NMR spectra were recorded on a Brüker 500

(500 MHz) spectrometer (chemical shifts are given in ppm (δ) relative to TMS as internal standard, coupling constants (J) are in hertz (Hz), and signals are using the following abbreviations: s, singlet; d, doublet; t, triplet; m, multiplet, etc. Mass spectra (MS), ESI (positive) were recorded on an Esquire-LC-00075 spectrometer. Thin layer chromatography was carried out using plate silica gel F254 Merck. Reagents and solvents were purchased from common commercial suppliers and were used without further purification. All yields are unoptimized and generally represent the result of a single experiment.

4.1.1. Synthesis of 2-amino-4,5-bis(4-chlorophenyl)thiophene-3-carboxylic acid (4)

2 N aqueous NaOH (75 mL) was added to a solution of methyl 2-amino-4,5-bis (4-chlorophenyl)thiophene-3-carboxylate (**3**, 5 g, 13 mmol) in 125 mL ethanol. The resulting mixture was refluxed for 2 h. Upon cooling, the reaction mixture was evaporated under reduced pressure. The residue was suspended in H_2O, and the suspension was acidified to pH 6.0 with 1 N HCl aqueous solution. The mixture was extracted with ethyl acetate (2 × 200 mL) and organic layer was washed with water (2 × 200 mL) and brine (2 × 200 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to give the crude product as a yellow solid. Since compound **4** was not very stable, the crude product was used in the next step without purification.

4.1.2. General procedure for the synthesis of 3,4,5-trisubstituted aminothiophene derivatives (5–25)

2-Amino-4,5-bis(4-chlorophenyl)thiophene-3-carboxylic acid (**4**, 0.4 mmol), EDCI (1.2 mmol) and HOBt (0.8 mmol) were dissolved in anhydrous CH_2Cl_2 (20 mL) and stirred for 30 min. Then the corresponding amine (0.8 mM) was added dropwise. After stirred overnight at room temperature, the reaction mixture was washed with water (2 × 20 mL) and brine (2 × 20 mL), and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue obtained was purified by silica gel column chromatography (petroleum ether/ethyl acetate/CH_2Cl_2 = 10:1:1–1:1:1 v/v/v) to get target compounds **5–25**.

4.1.2.1. 2-Amino-4,5-bis(4-chlorophenyl)-*N*-cyclobutylthiophene-3-carboxamide (6). Yellow solid (73%), mp: 175–177 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.41 (d, J = 8.1 Hz, 2H, Ar-H), 7.23 (d, J = 8.1 Hz, 2H, Ar-H), 7.12 (d, J = 8.3 Hz, 2H, Ar-H), 6.91 (d, J = 8.3 Hz, 2H, Ar-H), 6.35 (br s, 2H, NH_2), 4.92 (d, J = 7.0 Hz, 1H, NH), 4.25 (dd, J = 15.9, 8.0 Hz, 1H, CH), 2.17 (m, 2H, CH_2), 1.61 (m, 2H, CH_2), 1.32 (m, 2H, CH_2). ESI-MS: m/z = 417 $[M+1]^+$.

4.1.2.2. 2-Amino-4,5-bis(4-chlorophenyl)-*N*-cyclopentylthiophene-3-carboxamide (7). Yellow solid (69%), mp: 182–183 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.38 (d, J = 8.3 Hz, 2H, Ar-H), 7.22 (m, 4H, Ar-H), 6.94 (d, J = 8.5 Hz, 2H, Ar-H), 6.43 (br s, 2H, NH_2), 6.32 (d, J = 6.8 Hz, 1H, NH), 4.63 (m, 1H, CH), 1.90 (m, 2H, CH_2), 1.54 (m, 2H, CH_2), 1.35 (m, 2H, CH_2), 1.00 (m, 2H, CH_2). ESI-MS: m/z = 431 $[M+1]^+$.

4.1.2.3. 2-Amino-4,5-bis(4-chlorophenyl)-*N*-(4-hydroxycyclohexyl)thiophene-3-carboxamide (9). Yellow solid (55%), mp: 221–223 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38 (d, J = 8.4 Hz, 2H, Ar-H), 7.21 (d, J = 8.4 Hz, 2H, Ar-H), 7.11 (d, J = 8.5 Hz, 2H, Ar-H), 6.89 (d, J = 8.6 Hz, 2H, Ar-H), 6.32 (br s, 2H, NH_2), 4.65 (d, J = 7.3 Hz, 1H, NH), 4.57 (s, 1H, OH), 3.65 (m, 1H, CH), 3.49 (m, 1H, CH), 1.76 (m, 4H, $2CH_2$), 1.31 (m, 2H, CH_2), 0.70 (m, 2H, CH_2). ESI-MS: m/z = 461 $[M+1]^+$.

4.1.2.4. 2-Amino-4,5-bis(4-chlorophenyl)thiophen-3-yl)(piperidin-1-yl)methanone (11). White solid (83%), mp: 188–189 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.25 (d, J = 8.5 Hz, 2H, Ar-H), 7.18 (d, J = 8.5 Hz, 2H, Ar-H), 7.12 (d, J = 8.5 Hz, 2H, Ar-H), 7.01 (d, J = 8.5 Hz, 2H, Ar-H), 3.31 (m, 4H, $2CH_2$), 1.42 (m, 6H, $3CH_2$). ESI-MS: m/z = 431 $[M+1]^+$.

4.1.2.5. 2-Amino-4,5-bis(4-chlorophenyl)thiophen-3-yl)(4-methylpiperidin-1-yl)methanone (12). Yellow solid (68%), mp: 188–190 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.24 (d, J = 7.2 Hz, 1H, Ar-H), 7.21 (d, J = 8.1 Hz, 1H, Ar-H), 7.17 (d, J = 6.9 Hz, 2H, Ar-H), 7.13 (d, J = 8.2 Hz, 1H, Ar-H), 7.07 (d, J = 8.1 Hz, 1H, Ar-H), 7.01 (d, J = 7.9 Hz, 2H, Ar-H), 2.64 (m, 4H, $2CH_2$), 1.49 (m, 1H, CH), 1.36 (m, 2H, CH_2), 1.26 (m, 2H, CH_2), 0.68 (d, J = 6.2 Hz, 3H, CH_3). ESI-MS: m/z = 445 $[M+1]^+$.

4.1.2.6. 1-(2-Amino-4,5-bis(4-chlorophenyl)thiophene-3-carbonyl)piperidin-2-one (13). Yellow solid (48%), mp: 252–254 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.24 (d, J = 8.4 Hz, 2H, Ar-H), 7.18 (d, J = 8.5 Hz, 2H, Ar-H), 7.10 (d, J = 8.4 Hz, 2H, Ar-H), 6.99 (d, J = 8.5 Hz, 2H, Ar-H), 4.01 (m, 2H, CH_2), 3.74 (m, 2H, CH_2), 3.23 (m, 2H, CH_2), 2.83 (m, 2H, CH_2). ESI-MS: m/z = 445 $[M+1]^+$.

4.1.2.7. 1-(2-Amino-4,5-bis(4-chlorophenyl)thiophene-3-carbonyl)piperidin-4-one (14). Yellow solid (40%), mp: 290–293 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.24 (d, J = 8.3 Hz, 2H, Ar-H), 7.18 (d, J = 8.2 Hz, 2H, Ar-H), 7.13 (d, J = 8.1 Hz, 2H, Ar-H), 6.99 (d, J = 8.4 Hz, 2H, Ar-H), 3.62 (m, 2H, CH_2), 3.43 (m, 2H, CH_2), 2.24 (m, 2H, CH_2), 1.87 (m, 2H, CH_2). ESI-MS: m/z = 445 $[M+1]^+$.

4.1.2.8. 1-(2-Amino-4,5-bis(4-chlorophenyl)thiophene-3-carbonyl)piperidine-2,4-dione (15). Yellow solid (43%), mp: 261–263 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37 (d, J = 8.3 Hz, 2H, Ar-H), 7.24 (d, J = 8.3 Hz, 2H, Ar-H), 7.12 (d, J = 8.5 Hz, 2H, Ar-H), 6.89 (d, J = 8.5 Hz, 2H, Ar-H), 6.46 (br s, 2H, NH_2), 4.90 (m, 2H, CH_2), 3.43 (m, 2H, CH_2), 1.65 (m, 2H, CH_2). ESI-MS: m/z = 459 $[M+1]^+$.

4.1.2.9. (2-Amino-4,5-bis(4-chlorophenyl)thiophen-3-yl)(morpholino)methanone (16). White solid (80%), mp: 186–187 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.26 (d, J = 8.4 Hz, 2H, Ar-H), 7.17 (d, J = 8.4 Hz, 2H, Ar-H), 7.09 (d, J = 8.4 Hz, 2H, Ar-H), 6.98 (d, J = 8.4 Hz, 2H, Ar-H), 3.44 (m, 8H, $4CH_2$). ESI-MS: m/z = 433 $[M+1]^+$.

4.1.2.10. (2-Amino-4,5-bis(4-chlorophenyl)thiophen-3-yl)(piperazin-1-yl)methanone (17). Yellow solid (50%), mp: 182–183 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.24 (d, J = 8.4 Hz, 2H, Ar-H), 7.17 (dd, J = 8.8, 2.2 Hz, 2H, Ar-H), 7.09 (d, J = 8.4 Hz, 2H, Ar-H), 6.99 (dd, J = 8.4, 6.6 Hz, 2H, Ar-H), 4.57 (s, 1H, NH), 3.25 (m, 4H, $2CH_2$), 2.2 (m, 4H, $2CH_2$). ESI-MS: m/z = 432 $[M+1]^+$.

4.1.2.11. (2-Amino-4,5-bis(4-chlorophenyl)thiophen-3-yl)(4-methylpiperazin-1-yl)methanone (18). Yellow solid (52%), mp: 184–186 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.24 (d, J= 8.4 Hz, 2H, Ar-H), 7.18 (d, J = 8.5 Hz, 2H, Ar-H), 7.09 (d, J = 8.3 Hz, 2H, Ar-H),6.98 (d, J = 8.4 Hz, 2H, Ar-H), 3.23 (m, 4H, $2CH_2$), 2.01 (s, 3H, CH_3), 1.67 (m, 4H, $2CH_2$). ESI-MS: m/z = 446 $[M+1]^+$.

4.1.2.12. (2-Amino-4,5-bis(4-chlorophenyl)thiophen-3-yl)(3-methylpiperazin-1-yl)methanone (19). Yellow solid (47%), mp: 184–187 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.25 (d, J = 7.9 Hz, 2H, Ar-H), 7.16 (d, J = 8.5 Hz, 2H, Ar-H), 7.08 (d, J = 8.5 Hz, 2H, Ar-H), 6.98 (d, J = 7.7 Hz, 2H, Ar-H), 4.62 (m, 2H, CH_2), 3.34 (m, 1H, CH), 2.80 (m, 3H, CH_2), 2.44 (m, 1H, CH_2), 1.13 (m, 3H, CH_3). ESI-MS: m/z = 446 $[M+1]^+$.

4.1.2.13. 2-Amino-*N*-butyl-4,5-bis(4-chlorophenyl)thiophene-3-carboxamide (20). Yellow solid (63%), mp: 157–159 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38 (d, J = 8.5 Hz, 2H, Ar-H), 7.22 (d, J = 8.3 Hz, 2H, Ar-H), 7.11 (d, J = 8.9 Hz, 2H, Ar-H), 6.90 (d, J = 8.5 Hz, 2H, Ar-H), 4.83 (s, 1H, NH), 3.10 (dd, J = 12.2, 6.7 Hz, 2H, CH_2), 1.13 (dt, J = 14.8, 6.9 Hz, 2H, CH_2), 1.05 0.93 (m, 2H, CH_2), 0.74 (t, J = 7.0 Hz, 3H, CH_3). ESI-MS: m/z = 419 $[M+1]^+$.

4.1.2.14. 2-Amino-4,5-bis(4-chlorophenyl)-*N*-isobutylthiophene-3-carboxamide (21). Yellow solid (70%), mp: 151–153 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.41 (d,J = 8.5 Hz, 2H, Ar-H), 7.23 (d,J = 6.5 hz, 2H, Ar-H), 7.13 (d, J = 6.4 hz, 2H, Ar-H), 6.91 (d, J = 8.4 Hz, 2H, Ar-H), 6.32 (br s, 2H, NH_2), 4.93 (s, 1H, NH), 3.00 (m, 2H, CH_2), 1.49 (m, 1H, CH), 0.68 (m, 6H, $2CH_3$). ESI-MS: m/z = 419 $[M+1]^+$.

4.1.2.15. 2-Amino-*N*-(*tert*-butyl)-4,5-bis(4-chlorophenyl)thiophene-3-carboxamide (22). Yellow solid (66%), mp: 187–189 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.41(d,J = 8.4 Hz, 2H, Ar-H), 7.23 (d,J = 6.4 Hz, 2H, Ar-H), 7.14 (d, J = 6.5 Hz, 2H, Ar-H), 6.93(d, J = 8.4 Hz, 2H, Ar-H), 6.77 (br s 2H, NH_2), 4.72 (s, 1H, NH), 1.06 (s, 9H, $3CH_3$). ESI-MS: m/z = 419 $[M+1]^+$.

4.1.2.16. 2-Amino-*N*-benzyl-4,5-bis(4-chlorophenyl)thiophene-3-carboxamide (24). White solid (81%), mp: 213–215 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.26 (m, 3H, Ar-H),7.19 (d, J = 8.3 Hz, 2H, Ar-H), 7.12 (d, J = 7.5 Hz, 2H, Ar-H), 7.10 (d, J = 8.7 Hz, 2H, Ar-H), 6.92 (d, J = 7.2 Hz, 2H, Ar-H), 6.88 (d, J = 8.6 Hz, 2H, Ar-H), 5.06 (d, J = 3.7 Hz, 1H, NH), 4.26 (d, J = 4.0 Hz, 2H, CH_2). ESI-MS: m/z = 453 $[M+1]^+$.

4.1.2.17. 2-Amino-4,5-bis(4-chlorophenyl)-*N*-(4-methoxybenzyl)thiophene-3-carboxamide (25). White solid (77%), mp: 204–206 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.30 (d, J = 8.3 Hz, 2H, Ar-H), 7.23 (d, J = 8.5 Hz, 2H, Ar-H), 7.16 (d, J = 8.3 Hz, 2H, Ar-H), 7.02 (d, J = 7.8 Hz, 2H, Ar-H), 6.93 (d, J = 8.5 Hz, 2H, Ar-H), 6.77 (d, J = 7.8 Hz, 2H, Ar-H), 6.18 (t, J = 5.3 Hz, 1H, NH), 4.11 (d, J = 5.5 Hz, 2H, CH_2), 2.26 (s, 3H, CH_3). ESI-MS: m/z = 483 $[M+1]^+$.

4.1.3. Synthesis of *N*-(4,5-bis(4-chlorophenyl)-3-(piperidine-1-carbonyl)thiophen-2-yl)acetamide (26)

A solution of acetyl chloride (37 mg, 0.48 mmol) in anhydrous CH_2Cl_2 (5 mL) was slowly added to a cooled (0 °C) mixture of **11** (150 mg, 0.32 mmol) and triethylamine (0.96 mmol) in anhydrous CH_2Cl_2 (10 mL). After stirred at room temperature for additional 1 h, the reaction was quenched with water (10 mL). The mixture was extracted with CH_2Cl_2 (2 × 15 mL). The organic phase was washed with water (2 × 15 mL) and brine (2 × 15 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The obtained residue was purified by silica gel column chromatography (petroleum ether/ethyl acetate = 5:1, v/v) to give a white solid **26** (98%). mp: 261–263 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.40 (s, 1H, NH), 7.25 (m, 4H, Ar-H), 7.11 (m, 4H, Ar-H), 3.47 (m, 2H, CH_2), 2.65 (m, 2H, CH_2), 2.23 (s, 3H,CH_3), 2.08 (m, 2H, CH_2), 1.67 (m, 2H, CH_2), 1.49 (m, 2H, CH_2). ESI-MS: m/z = 473 $[M+1]^+$.

4.1.4. Synthesis of *N*-(4,5-bis(4-chlorophenyl)-3-(piperidine-1-carbonyl)thiophen-2-yl)-3-morpholinopropanamide (27)

A solution of chloropropionyl chloride (60 mg, 0.48 mmol) in anhydrous CH_2Cl_2 (5 mL) was slowly added to a cooled (0 °C) mixture of **11** (150 mg, 0.32 mmol) and triethylamine (0.96 mmol) in anhydrous CH_2Cl_2 (10 mL). After stirred at room temperature for additional 1 h, the reaction was quenched with water (10 mL). The mixture was extracted with CH_2Cl_2 (2 × 15 mL). The organic phase was washed with water (2 × 15 mL) and brine (2 × 15 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. Then, the obtained residue was dissolved in 10 mL dioxane, to which was added triethylamine (1.6 mmol) and morpholine (139 mg, 3.2 mmol), refluxed for 1 h. Upon cooling, the reaction mixture was evaporated under reduced pressure and the residue obtained was dissolved in ethyl acetate. The organic phase was washed with water (2 × 15 mL) and brine (2 × 15 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The obtained residue was purified by silica gel column chromatography (petroleum ether/ethyl acetate = 1:3, v/v) to give a yellow solid **27** (69%). mp: 178–180 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.40 (s, 1H, NH), 7.25 (m, 4H, Ar-H), 7.19 (m, 4H, Ar-H), 4.08 (m, 4H, $2CH_2$), 3.63 (m, 4H, $2CH_2$), 2.76 (m, 4H, $2CH_2$), 2.63 (m, 2H, CH_2), 2.59 (m, 2H, CH_2) 1.54 (m, 6H, $3CH_2$). ESI-MS: m/z = 572 $[M+1]^+$.

4.1.5. Synthesis of 4,5-bis(4-chlorophenyl)-*N*-cyclopropyl-2-(methylsulfonamido)thiophene-3-carboxamide (28)

A solution of methanesulfonyl chloride (64 mg, 0.56 mmol) in anhydrous CH_2Cl_2 (5 mL) was slowly added to a cooled (0 °C) mixture of **5** (150 mg, 0.37 mmol) and triethylamine (1.11 mmol) in anhydrous CH_2Cl_2 (10 mL). After stirred at room temperature for additional 1 h, the reaction was quenched with water (10 mL). The mixture was extracted with CH_2Cl_2 (2 × 15 mL). The organic phase was washed with water (2 × 15 mL) and brine (2 × 15 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The obtained residue was purified by silica gel column chromatography (petroleum ether/ethyl acetate = 5:1, v/v) to give a yellow solid **28** (100%). mp: 191–193 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.29 (d, J = 8.4 Hz, 2H, Ar-H), 7.23 (d, J = 8.5 Hz, 2H, Ar-H), 7.14 (d, J = 8.4 Hz, 2H, Ar-H), 7.08 (d, J = 8.5 Hz, 2H, Ar-H), 6.03 (d, J = 2.0 Hz, 1H, NH), 3.54 (s, 3H, CH_3), 2.65 (tq, J = 7.2, 3.7 Hz, 1H, CH), 0.77 (m, 2H, CH_2), 0.36 (m, 2H, CH_2). ESI-MS: m/z = 481 $[M+1]^+$.

4.1.6. Synthesis of ethyl (4,5-bis(4-chlorophenyl)-3-(cyclopropylcarbamoyl)thiophen-2-yl)carbamate (29)

A solution of ethyl carbonochloridate (78 mg, 0.74 mmol) in toluene (5 mL) was added to a mixture of **5** (150 mg, 0.37 mmol) and anhydrous potassium carbonate (255 mg, 1.85 mmol) in dry toluene (10 mL). After stirred at room temperature for additional 3 h, toluene was removed under reduced pressure and the obtained residue was dissolved in ethyl acetate (20 mL), washed with water (2 × 20 mL) and brine (2 × 20 mL). The organic phase was dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The obtained residue was purified by silica gel column chromatography (petroleum ether/ethyl acetate = 2:1, v/v) to give a yellow solid **29** (82%). mp: 151–153 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.40 (s, 1H, NH), 7.39 (d, J = 8.3 Hz, 2H, Ar-H), 7.16 (m, 4H, Ar-H), 6.98 (d, J = 8.5 Hz, 2H, Ar-H), 5.13 (s, 1H, NH), 4.29 (q, J = 7.1 Hz, 2H, CH_2), 2.61 (m, 1H, CH), 1.33 (t, J = 7.2 Hz, 3H, CH_3), 0.66 (m, 2H, CH_2), 0.05 (m, 2H, CH_2). ESI-MS: m/z = 475 $[M+1]^+$.

4.1.7. Synthesis of 4,5-bis(4-chlorophenyl)-*N*-cyclopropyl-2-(3-(2-morpholinoethyl)ureido)thiophene-3-carboxamide (30)

A solution of **5** (150 mg, 0.32 mmol) in anhydrous CH_2Cl_2 (10 mL) was added triethylamine (0.64 mmol) and 2-chloroethyl isocyanate (506 mg, 4.8 mmol). The mixture was refluxed overnight. The reaction was quenched with water (10 mL). The mixture was extracted with CH_2Cl_2 (2 × 15 mL). The organic phase was washed with water (2 × 15 mL) and brine (2 × 15 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. Then, the obtained residue was dissolved in 10 mL dioxane, to which was added triethylamine (1.6 mmol) and morpholine (139 mg, 3.2 mmol), refluxed for 1 h. After cooling to room temperature, the reaction mixture was evaporated under reduced pressure. The residue was extracted with ethyl acetate (2 × 15 mL).

The organic phase was washed with water (2 × 15 mL) and brine (2 × 15 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The obtained residue was purified by silica gel column chromatography (petroleum ether/ethyl acetate = 1:5, v/v) to give a yellow solid **30** (56%). mp: 129–130 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.60 (s, 1H, NH), 7.39 (d, J = 8.4 Hz, 2H, Ar-H), 7.16 (d, J = 8.4 Hz, 2H, Ar-H), 7.15 (d, J = 8.4 Hz, 2H, Ar-H), 6.99 (d, J = 8.4 Hz, 2H, Ar-H), 5.73 (br s 1H, NH), 5.16 (s, 1H, NH), 3.82 (m, 4H, $2CH_2$), 3.51 (m, 2H, CH_2), 2.66 (m, 2H, CH_2), 2.64 (m, 4H, $2CH_2$), 2.47 (m, 1H, CH), 0.74 (m, 2H, CH_2), 0.05 (m, 2H, CH_2). ESI-MS: m/z = 559 $[M+1]^+$.

4.1.8. Synthesis of 4,5-bis(4-chlorophenyl)-*N*-cyclopropyl-2-(3-(2-(diethylamino)ethyl)ureido)thiophene-3-carboxamIde (31)

Same as **30** above, except with diethylamine. Target compound **31** was obtained as a yellow solid (50%). mp: 120–121 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.53 (s, 1H, NH), 7.37 (d, J = 8.1 Hz, 2H, Ar-H), 7.15 (d, J = 8.3 Hz, 2H, Ar-H), 7.13 (d, J = 8.5 Hz, 2H, Ar-H), 6.96 (d, J = 8.3 Hz, 2H, Ar-H), 6.74 (br s, 1H, NH), 5.09 (s, 1H, NH), 3.61 (m, 2H, CH_2), 2.94 (m, 6H, $3CH_2$), 2.60 (d, J = 3.3 Hz, 1H, CH), 1.25 (s, 6H, $2CH_3$), 0.63 (m, 2H, CH_2), 0.02 (m, 2H, CH_2). ESI-MS: m/z = 545 $[M+1]^+$.

4.2. Biological evaluation

4.2.1. MDM2 protein expression and purification

MDM2 (1–118) plasmid was provided by Dr. Shaomeng Wang's group, and transformed into Escherichia coli BL-21 (DE3). Cultures were grown at 37 °C in TB medium, and induced by 0.4 mM IPTG at an OD600 of 0.6 at 18 °C for 20 h. Cells were lysed in 50 mM Tries, pH 7.5 buffer containing 500 mM NaCl and 10% glycerol. MDM2 (1–118) was purified from the soluble fraction using Ni-NTA resin, and desalted in PBS buffer pH 7.5, 150 mM NaCl and 10% glycerol. The protein was purified to >95% as judged SDS–PAGE.

4.2.2. Fluorescence polarization competitive binding assay

Measurements were made with a Beckmancoulter DTX880 Multilabel Plate Reader using a 485 nM excitation filter and a 535 nM emission filter. Assays were performed in Microtiter 96-Well black and round bottom plates. Nutlin-3 was used as a positive control, while DMSO was used as a negative control. Assays were performed in duplicate and repeated at least three times on separate days. Competition experiments were carried out in a total volume of 20 μL 40 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 1 mM DTT, 4% DMSO. Probe peptide was present at a final concentration of 1 nM, and MDM2 was present at a final concentration of 10 nM. Plates were allowed to incubate at room temperature for 1 h prior to measurement. The K_i values for inhibitors were calculated by a web-based computer program.[17]

4.2.3. Cell proliferation assay

Cell proliferation was assessed by sulforhodamin B (SRB) assay. Briefly, A549 and PC3 cells were seeded into 96-well plates and cultured overnight and then exposed to serial concentrations of compounds for 72 h. Cells were then washed with PBS and fixed with 10% (w/v) trichloroacetic acid at 4 °C for an hour. After washing, the cells were stained for 30 min with 0.4% SRB dissolved in 1% acetic acid. Then the cells were washed by 1% acetic acid for 5 times, and protein-bound dye was extracted with 10 mmol unbuffered Tris base. The absorbance was measured at 515 nm using a multiscan spectrum (Thermo Electron Co., Vantaa, Finland). The inhibition rate on cell proliferation of each well was calculated as (A515 control cells− A515 treated cells)/A515 control cells × 100%. The average IC_{50} values were determined by Logit method from at least two independent tests.

4.3. Molecular docking

Docking simulations were carried out by using C-DOCKER module (Discovery Studio, version 2.1; Accelrys, San Diego, CA, USA, 2008). The X-ray crystal structure of MDM2 bound to the transactivation domain of p53 (PDB ID:1YCR) was used for the docking calculation. After removing the ligand and solvent molecules, the CHARMm-force field was applied to the protein. And the area around p53 peptide was chosen as the active site with a radius set as 10 Å. Each compound was generated random conformations using CHARMm-based molecular dynamics (1000 steps), and then docked into the defined MDM2 binding site. The other parameters were set as default. The final binding conformation of **3** and **24** was determined based on the calculated CDOCKING ENERAGE. The most stable binding mode among the top 10 docking poses of each compound was presented in Fig. 2, respectively.

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