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Discovery of novel leucyladenylate sulfamate surrogates as leucyl-tRNA synthetase (LRS)-targeted mammalian target of rapamycin complex 1 (mTORC1) inhibitors

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A B S T R A C T

According to recent studies, leucyl-tRNA synthetase (LRS) acts as a leucine sensor and modulates the activation of the mammalian target of rapamycin complex 1 (mTORC1) activation. Because overactive mTORC1 is associated with several diseases, including colon cancer, LRS-targeted mTORC1 inhibitors represent a potential option for anti-cancer therapy. In this work, we developed a series of simplified leucyladenylate sulfamate analogues that contain the *N*-(3-chloro-4-fluorophenyl)quinazolin-4-amine moiety to replace the adenine group. We identified several compounds with comparable activity to previously reported inhibitors and exhibited selective mTORC1 inhibition and anti-cancer activity. This study further supports the hypothesis that LRS is a promising target to modulate the mTORC1 pathway.

1. Introduction

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that plays a crucial role in cell metabolism, growth, proliferation, and autophagy. mTOR exists as two structurally and functionally different multi-protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 regulates protein synthesis by phosphorylating two major substrates, S6 kinase 1 (S6K1) and the translational regulators eukaryotic translation initiation factor 4E (elF4E)-binding protein 1 (4E-BP1), whereas mTORC2 regulates cell survival and metabolism.[1] Notably, overactive mTORC1 has recently been shown to be associated with various human diseases, such as diabetes, neurodegenerative diseases, and cancers.[2–4]

Rapamycin and its analogs are well known allosteric inhibitors of mTORC1 and have been widely studied for use as anti-cancer agents. Rapamycin and its derivatives, also called rapalogs, inhibit downstream phosphorylation by binding to the FK506-binding protein 12 (FKBP12) and interacting with the FKBP12-rapamycin binding (FRB) domain in mTORC1.[5] Although many rapalogs have been developed for anti-cancer therapy,[6,7] these agents appear to only partially inhibit mTORC1 activity, and thus are not sufficient to suppress mTORC1 activity in many pathological conditions. Moreover, partial suppression of mTORC1 often leads to rapamycin-resistance in cancer cells; therefore, small molecules targeting other possible regulators of the mTORC1 pathway represent an alternative strategy to overcome rapamycin resistance.

Leucine plays a major role in controlling the amino acid-dependent activation of the mTORC1 signaling pathway.[8,9] Although the exact mechanism by which leucine activates mTORC1 pathway has not been completely elucidated, several recent studies revealed that leucyl-tRNA synthetase (LRS) acts as an intracellular leucine sensor by directly binding to RagD GTPase, one of the key mediators of the amino acid-dependent mTORC1 signaling pathway.[10,11] LRS is a member of the class I aminoacyl-tRNA synthetase (ARSs) family that catalyzes the ATP-dependent ligation of leucine to the cognate tRNA during protein biosynthesis. Notably, LRS acts as a GTPase-activating protein (GAP) for Rag GTPase to activate mTORC1;[10] hence, inhibitors of LRS suppress the hydrolysis of RagD-GTP to RagD-GDP, blocking mTORC1 activity. Moreover, one leucine analogue, leucinol, blocks the amino acid-mediated activation of mTORC1 by inhibiting the leucine-sensing

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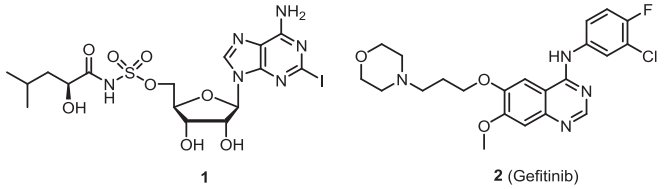


Fig. 1. Structures of (*S*)-2-hydroxy-4-methylpentanoyl adenylate sulfamate (**1**) and gefitinib (**2**).

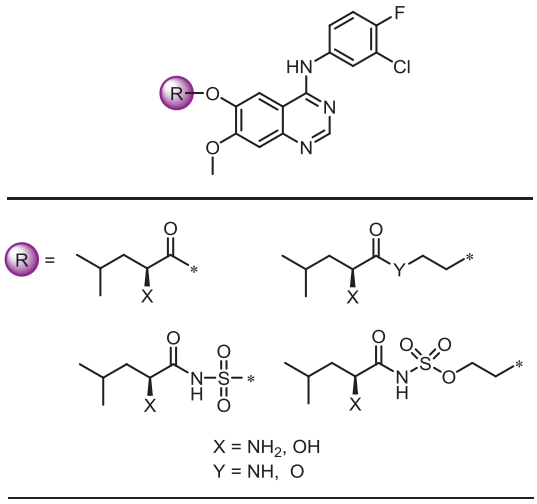


Fig. 2. Newly designed simplified leucyladenylate analogues.

ability of LRS without affecting its catalytic function.[12,13]

As shown in our recent study, (*S*)-2-hydroxy-4-methylpentanoyl adenylate sulfamate (**1**) is a leucyladenylate sulfamate surrogate that selectively inhibits LRS-mediated mTORC1 activation without affecting the catalytic activity of LRS. Compound **1** also exhibits specific anti-tumor activity against colon cancer cells expressing hyperactive mTORC1, suggesting that LRS-targeted mTORC1 inhibitors may represent a novel treatment option for human colorectal cancer.[14] In addition, simplified analogues of the leucyladenylate sulfamates maintain specific binding to LRS and exhibit improved lipophilicity and better synthetic accessibility.[15]

In our continuing efforts to develop LRS-targeted mTORC1 inhibitors as potential anti-cancer agents, we aim to expand the library of our simplified leucyladenylate analogues by exploring a hybrid scaffold combining the structures of **1** and gefitinib (**2**, Iressa) (Fig. 1). Gefitinib is a selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor that binds to the ATP-binding site of the kinase domain.[16] This new series of simplified leucyladenylate analogues contain the *N*-(3-chloro-4-fluorophenyl)quinazolin-4-amine moiety of gefitinib, which represents the adenine-mimicking group, combined with leucine surrogates through various linkers, replacing the 5-*O*-sulfamoylribose group (Fig. 2).

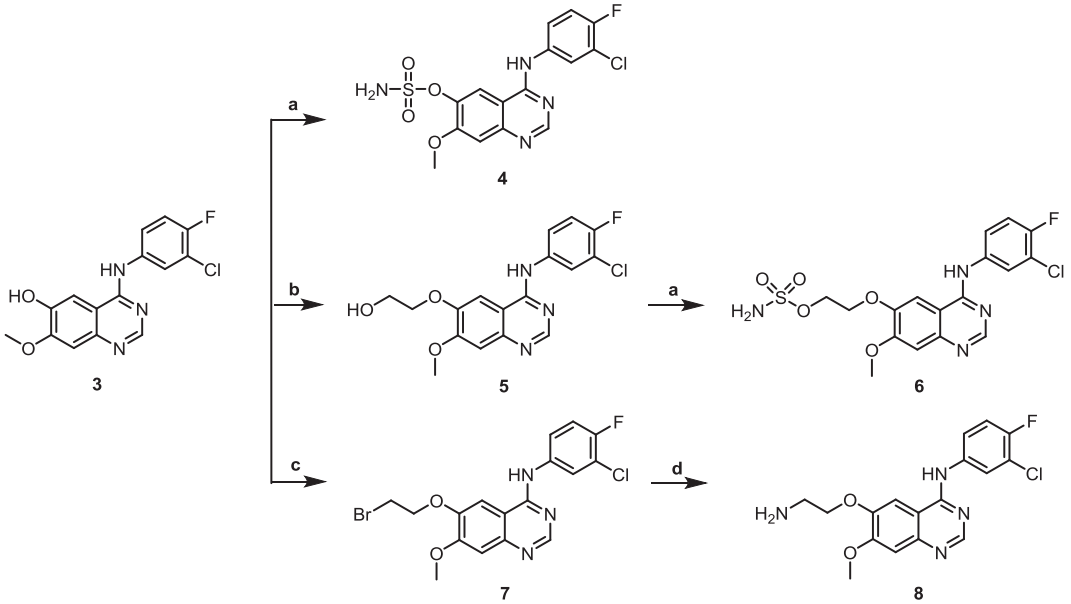
Here, we describe the synthesis of newly designed compounds and their inhibitory effects on the mTORC1 signaling pathway. In addition, we evaluated leucylation activity of selected mTORC1 inhibitors to investigate their mechanism of action and their cytotoxicities in various types of cancer cells to assess their anti-cancer activity.

2. Results and discussion

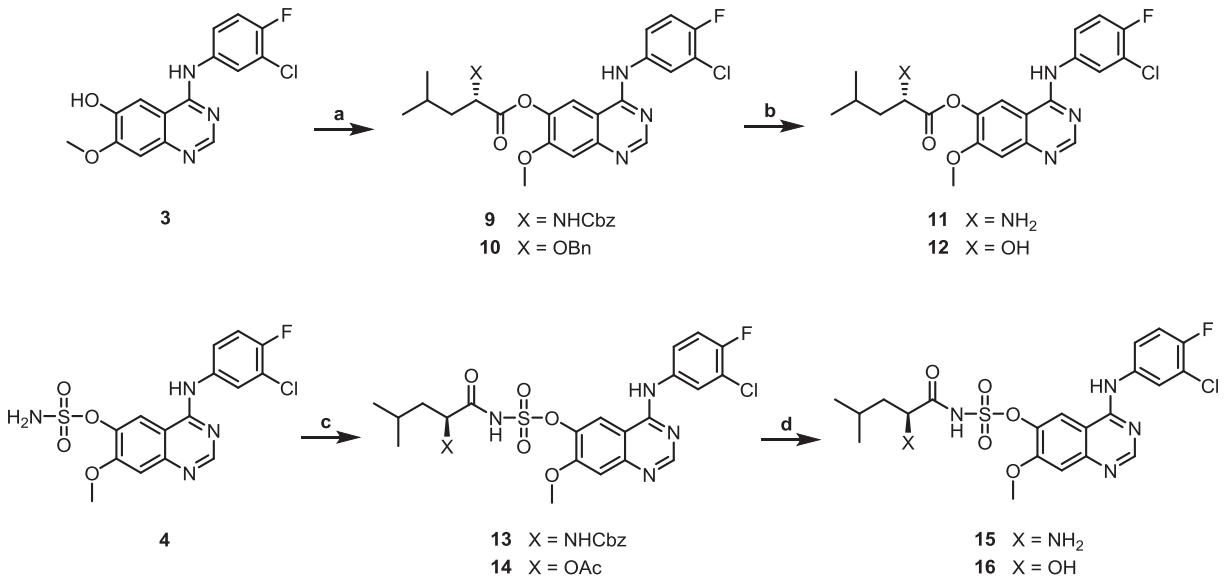
2.1. Chemistry

The syntheses of the *N*-(3-chloro-4-fluorophenyl)quinazolin-4-amine containing fragments (**4**, **5**, **6** and **8**) began with compound **3,** which was prepared using previously reported procedures[17] (Scheme 1). Compound **3** was reacted with sulfamoyl chloride prepared in a quantitative yield from chlorosulfonyl isocyanate according to a previously reported procedure to synthesize intermediate **4.**[18] A hydroxyethyl linker was introduced under basic conditions to yield intermediate **5**, which was then subsequently sulfamoylated to generate intermediate **6**. *O*-alkylation of compound **3** with 1,2-dibromoethane produced compound **7**, which was then reacted with an aqueous ammonia solution to produce a primary amino group.

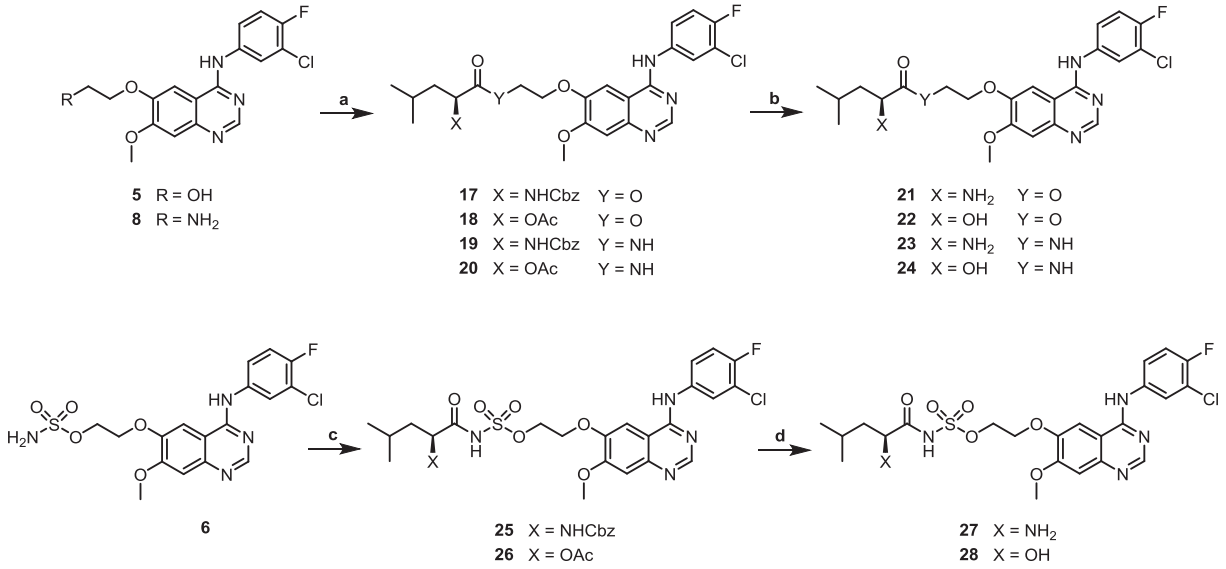
The compounds lacking the ethyl linker (**11, 12, 15** and **16**) were synthesized using the method illustrated in Scheme 2. Compound **3** was coupled with *N*-Cbz leucine and *O*-benzyl-protected (2*S*)-hydroxyisocaproic acid (HICA, *l*-leucic acid) to afford compounds **9** and **10**, respectively. Compound **4** was conjugated with *N*-Cbz leucine or *O*-acetyl-protected HICA to produce compounds **13** and **14**, respectively.



Scheme 1. Synthesis of the *N*-(3-chloro-4-fluorophenyl)quinazolin-4-amine-containing intermediates. *Reagents & conditions*: (a) sulfamoyl chloride, DMA, 0 °C to r.t., overnight; (b) 2-bromoethanol, K_2CO_3, DMF, r.t., 12 h; (c) 1,2-dibromoethane, K_2CO_3, DMF, r.t., 12 h; (d) NH_4OH, DMF, 50 °C, 24 h.



Scheme 2. Synthesis of the hybrid analogues **11, 12, 15**, and **16**. *Reagents & conditions*: (a) cyanuric chloride, TEA, acetone, *N*-Cbz leucine for **9**, *O*-benzyl-protected L-leucic acid for **10**, 0 °C to r.t., 2 h; (b) Pd/C, H_2, 2 M NH_3 in MeOH, r.t., overnight for **11**, BBr_3, CH_2Cl_2, -78 °C to r.t., 12 h for **12**; (c) DCC, DMAP, CH_2Cl_2, *N*-Cbz leucine for **13**, *O*-acetyl-protected L-leucic acid for **14,** r.t., 2 h; (d) Pd/C, H_2, 2 M NH_3 in MeOH, r.t., overnight for **15**, 0.02 M NaOMe, 0 °C to r.t., 2 h for **16.**



Scheme 3. Synthesis of the hybrid analogues with an ethyl linker, **21**–**24, 27**, and **28**. *Reagents & conditions*: (a) cyanuric chloride, TEA, acetone, *N*-Cbz leucine for **17** and **19**, *O*-acetyl-protected L-leucic acid for **18** and **20,** 0 °C to r.t., 2 h; (b) Pd/C, H_2, 2 M NH_3 in MeOH, r.t., overnight for **21** and **23**, 0.02 M NaOMe, 0 °C to r.t., 2 h for **22** and **24**; (c) DCC, DMAP, CH_2Cl_2, *N*-Cbz leucine for **25**, *O*-acetyl-protected L-leucic acid for **26**, r.t., 2 h; (d) Pd/C, H_2, 2 M NH_3 in MeOH, r.t., overnight for **27**, 0.02 M NaOMe, 0 °C to r.t., 2 h for **28**.

The *O*-benzyl- or *O*-acetyl-protected HICA were prepared by benzylation or acetylation of commercially available l-leucic acid, respectively. Deprotection of the α-amino or α-hydroxyl group of compounds **9, 10, 13** and **14** yielded the final compounds **11, 12, 15** and **16**, respectively.

Compounds with an ethyl linker (**21**–**24**, **27** and **28**) were synthesized from the *N*-(3-chloro-4-fluorophenyl)quinazolin-4-amine-containing fragments using the methods shown in Scheme 3. Compounds **17**–**28** were synthesized using the same procedures described in Scheme 2.

2.2. Biological activity

We examined the effects of the synthesized compounds on the mTORC1 pathway by determining the leucine-induced phosphorylation of S6K, a major mTORC1 substrate, using Western blotting. We screened our compounds by employing the cell-based kinase assay used in our previous studies.[14,15,19] We pretreated HEK293 cells with each final compound at one fixed concentration (200 μM) together with rapamycin (100 nM) and leucinol (800 μM) for comparison, and then activated mTORC1 by treating the cells with leucine for 10 min. As shown in Fig. 3, the rapamycin and leucinol pretreatment blocked leucine-induced S6K phosphorylation, as the phosphorylated S6K band (pS6K) displayed a weak intensity. Among the hybrid analogues without an ethyl spacer, compounds with a sulfonamide linker (**15** and **16**) exhibited comparable activity to leucinol, whereas compounds with an ester linker (**11** and **12**) showed no activity. In contrast, the hybrid analogs with an ethyl spacer displayed an opposite trend, in which the analogs with an ester or an amide linker (**21**–**24**) generally showed more potent activity, whereas compounds with a sulfonamide linker (**27** and **28**) had completely lost their activities, suggesting that the

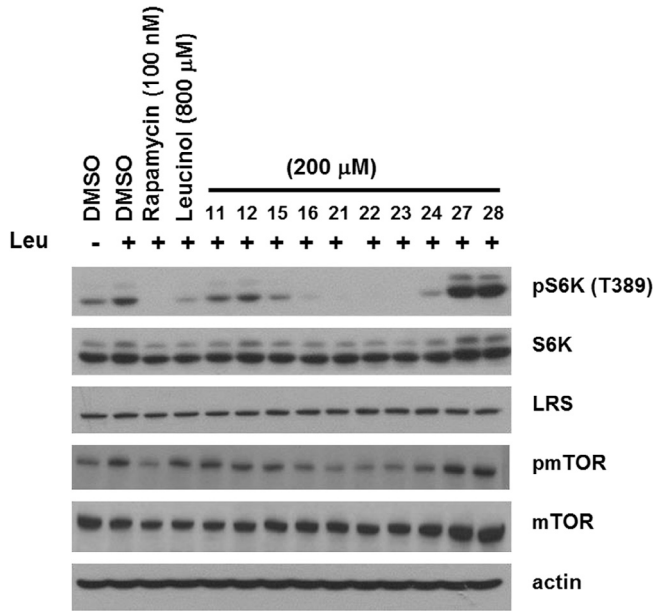


Fig. 3. Inhibition of leucine-induced mTORC1 activation in HEK293 cells treated with fixed concentrations of each compound.

distance between the leucyl side chain and the adenine binding site is critical for mTORC1 inhibition. Additionally, for compounds with a sulfonamide linker, compound **16**, which has α-hydroxyl group, showed more potent inhibition than its α-amino group-containing counterpart (**15**); however, for compounds with an ethyl amino linker, the compound with an α-amino group (**23**) showed more potent inhibition than the compound with an α-hydroxyl group (**24**), suggesting that the binding interactions may be altered by the linker structures, likely involving hydrogen-bond formation. When we examined the expression levels of other related proteins, such as S6K, LRS, mTOR, and pmTOR, their levels were consistent in cells treated with each of the compounds, supporting our hypothesis that these compounds suppressed the mTOR pathway by selectively interacting with LRS.

Next, we selected compounds **16, 21, 22,** and **23**, which appeared to be more potent than leucinol, and determined the dose-dependent inhibition of mTORC1 (Fig. 4). Compounds **16** and **22** inhibited S6K

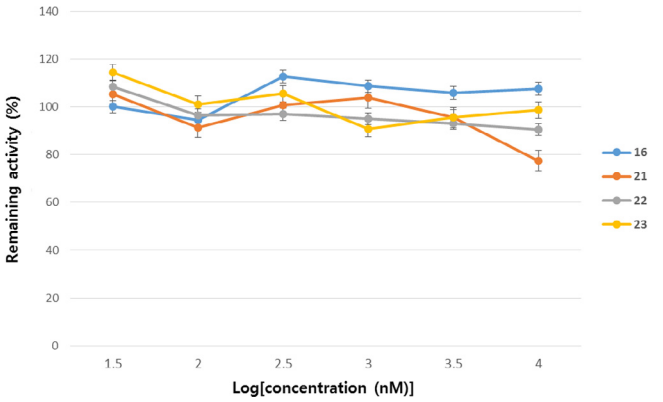


Fig. 5. Inhibition of catalytic leucylation by compounds **16**, **21**, **22** and **23**.

phosphorylation in a dose-dependent manner to similar extents, whereas compound **23** only inhibited the phosphorylation when applied at 200 μM. Compounds **16**, **21** and **22** strongly inhibited S6K phosphorylation at 100 μM, showing a significant reduction in pS6K band intensities, but compound **23** did not inhibit S6K phosphorylation at the same concentration. Because compounds **16** and **22** appeared to be the most potent compounds based on the band intensities, the hydroxyl group in the leucyl side chain may be crucial for activity. On the other hand, the cellular expression levels of S6K, LRS, mTOR, and pmTOR were not significantly altered by these compounds at any concentrations.

We performed aminoleucylation assays with compounds **16**, **21**, **22** and **23** to further investigate the mechanism by which these hybrid analogs inhibit the catalytic activity of LRS. As shown in Fig. 5, none of the tested compounds exerted a significant effect on leucylation activity when applied in the low micromolar range. In this case, > 90% of the enzyme activity remained, except for the reaction with compound **21**. Based on this result, these compounds selectively acted on the LRS-associated mTORC1 pathway in the same manner as leucine and leucinol without significantly affecting the catalytic activity of LRS, supporting our hypothesis that these simplified adenylate analogues selectively bind to LRS and suppress LRS-mediated mTORC1 activation.

We next performed the sulforhodamine B (SRB) colorimetric assays to assess the anticancer activity of compounds **16**, **21**, **22** and **23**.[20] Compounds **16**, **21**, **22** and **23** were administered to six different types

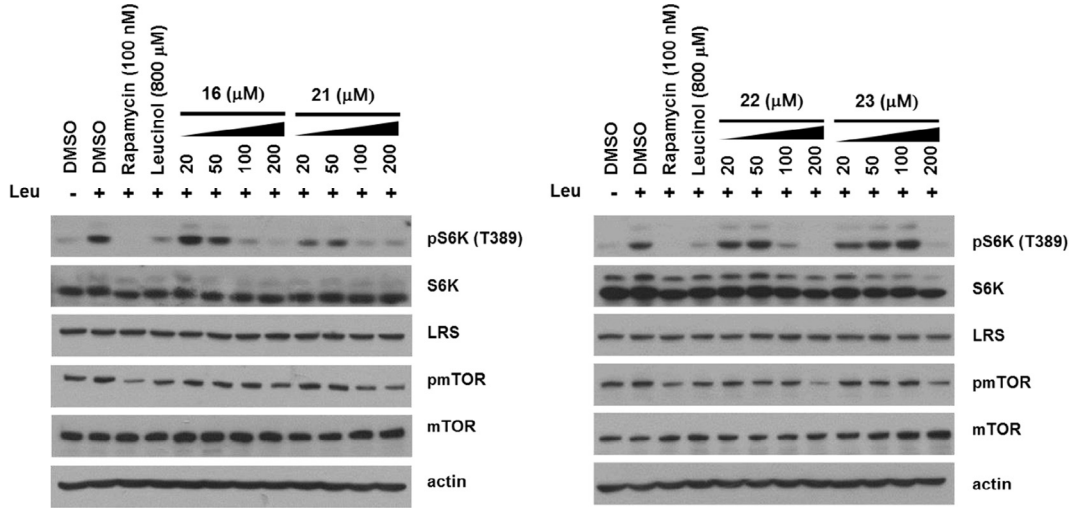


Fig. 4. Dose-dependent inhibition of leucine-induced mTORC1 activation in HEK293 cells by compounds **16**, **21**, **22** and **23**.

of cancer cell lines in parallel with etoposide as a positive control. As shown in Table 1, all compounds showed moderate cytotoxicity, displaying IC_{50} values in the low micromolar range against various cancer cell lines. Interestingly, when tested against the normal cell line, MRC5, compounds **16** and **23** showed little or no cytotoxicity, whereas compounds **21** and **22** exhibited a similar level of cytotoxicity as in cancer cells. We postulate that the cytotoxicity of compounds **21** and **22** is probably due to the inhibition of the catalytic activity of LRS. Overall, these observations supported the hypothesis that the specific inhibition of LRS-mediated activation of the mTOR pathway resulted in selective cytotoxicity towards cancer cells.

3. Conclusions

In summary, we developed a new series of simplified leucyladenylate sulfamate analogues that inhibited LRS-mediated mTORC1 activation. In this new series, we introduced the *N*-(3-chloro-4-fluorophenyl)quinazolin-4-amine group to replace the adenine moiety and incorporated various linker structures to study SARs. Compound **16** contains a leucylquinazoline sulfamate and showed a comparable inhibitory effect to leucinol, whereas compounds **21–23**, which contain an ethyl spacer with an ester or an amide linkage between the leucyl side chain and the quinazoline group, also exerted potent inhibitory effects. These compounds did not affect the levels of other related proteins, such as LRS and mTOR, nor did they affect the catalytic activity of LRS. Furthermore, these compounds showed general cytotoxicity towards various cancer cell lines, suggesting that these agents may serve as a potential therapeutic agents for the treatment of cancer.

4. Experimental section

4.1. General

All chemical reagents were commercially available. Melting points were determined on a melting point Buchi B-540 apparatus and are uncorrected. Silica gel column chromatography was performed on silica gel 60, 230–400 mesh, Merck. ^1H and ^{13}C NMR spectra were recorded on a JEOL JNM-LA 300 at 300 MHz, Bruker Analytik, DE/AVANCE Digital 400 at 400 MHz and 100 MHz, Bruker Analytik, DE/AVANCE Digital 500 at 500 MHz, JEOL JNM-ECA-700 at 175 MHz, respectively. Chemical shifts are reported in ppm units with Me_4Si as a reference standard. Mass spectra were recorded on a VG Trio-2 GC–MS instrument and a 6460 Triple Quad LC–MS instrument. All final compounds were purified to > 95% purity, as determined by high-performance liquid chromatography (HPLC). HPLC was performed on an Agilent 1120 Compact LC (G4288A) instrument using an Agilent TC-C18 column (4.6 mm × 250 mm, 5 μm), λ = 250 nm, flow rate 1.0 mL/min, Mobile phase (60:40) buffer/acetonitrile (buffer: 5 mol% ammoinium acetate aqueous solution. According to the HPLC analyses, final compounds **16**, **21–23** showed a purity of ≥95%.

Table 1
Relative inhibitory effects of compounds **16**, **21**, **22** and **23** on the growth of various cancer cell lines.[a]

IC_{50} (μM)	A549	HCT116	K562	MDA-MB-231	SK-HEP-1	SNU638	MRC5
16	4.38	4.04	4.10	6.23	3.30	4.94	18.78
21	3.59	3.97	4.01	3.65	1.94	3.86	1.87
22	3.16	3.12	3.89	2.05	1.39	3.41	1.12
23	2.79	4.24	3.83	4.59	2.21	2.97	> 20
Etoposide	0.30	1.06	0.76	1.53	0.63	1.05	11.73

[a] A549, lung cancer cells; HCT116, colon cancer cells; K562, leukemia cells; MDA-MB-231, breast cancer cells; SK-Hep-1, liver cancer cells; SNU638, stomach cancer cells; MRC5, normal lung epithelial cells.

4.2. Procedure

4.2.1. 4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl sulfamate (4)

A solution of compound **3** (200 mg, 0.625 mmol) in dimethylacetamide (4 mL) was treated with freshly prepared sulfamoyl chloride (108 mg, 0.938 mmol) at 0 °C and gradually warmed to room temperature. After stirring for 2 h, the reaction mixture was diluted with H_2O and extracted with EtOAc several times. The combined organic layers was washed with H_2O, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by silica gel column chromatography to obtain compound **4** (187 mg) in 75% yield as a pale yellow solid.; ^1H NMR (300 MHz, DMSO) δ 8.88 (s, 1H), 8.56 (s, 1H), 7.95 (dd, 1H, J = 6.78, 2.22 Hz), 7.63 (m, 1H), 7.54 (t, 1H, J = 8.97 Hz), 7.25 (s, 1H), 3.99 (s, 3H); MS (ESI) m/z 399 $[M + H]^+$.

4.2.2. 2-((4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethan-1-ol (5)

A solution of compound **3** (200 mg, 0.625 mmol) in DMF (5 mL) was treated with K_2CO_3 (260 mg, 1.88 mmol) and 2-bromoethanol (234 mg, 1.88 mmol) and then stirred at room temperature for 12 h. The reaction mixture was diluted with H_2O and extracted with EtOAc several times. The combined organic layers was washed with H_2O, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by silica gel column chromatography to obtain compound **5** (68 mg) in 30% yield as a white solid.; ^1H NMR (300 MHz, CD_3OD) δ 8.42 (s, 1H), 8.04 (dd, 1H, J = 6.60, 2.58 Hz), 7.74 (s, 1H), 7.69–7.63 (m, 1H), 7.25 (t, 1H, J = 8.97 Hz), 7.18 (s, 1H), 4.48 (t, 1H, J = 6.06 Hz), 3.99 (s, 3H), 3.71 (t, 1H, J = 6.03 Hz); MS (ESI) m/z 364 $[M + H]^+$.

4.2.3. 2-((4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl sulfamate (6)

By following the procedure for the synthesis of **4**, compound **6** was prepared in 75% yield as a white solid.; ^1H NMR (300 MHz, CD_3OD) δ 8.36 (s, 1H), 7.91 (dd, 1H, J = 6.57, 2.55 Hz), 7.65 (s, 1H), 7.60–7.56 (m, 1H), 7.16 (t, 1H, J = 8.97 Hz), 7.09 (s, 1H), 4.49–4.46 (m, 2H), 4.38–4.35 (m, 2H), 3.91 (s, 3H); MS (ESI) m/z 443 $[M + H]^+$.

4.2.4. 6-(2-Bromoethoxy)-N-(3-chloro-4-fluorophenyl)-7-methoxyquinazolin-4-amine (7)

A solution of compound **3** (200 mg, 0.625 mmol) in DMF (5 mL) was treated with K_2CO_3 (260 mg, 1.88 mmol) and 1,2-dibromoethane (352 mg, 0.936 mmol) and then stirred at room temperature for 8 h. The reaction mixture was diluted with H_2O and extracted with EtOAc several times. The combined organic layers was washed with H_2O, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by silica gel column chromatography to obtain compound **5** (102 mg) in 45% yield as a white solid.; ^1H NMR (300 MHz, CD_3OD) δ 8.44 (s, 1H), 8.00 (dd, 1H, J = 6.60, 2.58 Hz), 7.74 (s, 1H), 7.69–7.63 (m, 1H), 7.25 (t, 1H, J = 8.97 Hz), 7.18 (s, 1H), 4.50 (t, 1H, J = 6.06 Hz), 4.00 (s, 3H), 3.81 (t, 1H, J = 6.03 Hz); MS (FAB) m/z 427 $[M + H]^+$.

4.2.5. 6-(2-Aminoethoxy)-N-(3-chloro-4-fluorophenyl)-7-methoxyquinazolin-4-amine (8)

A solution of compound **7** (100 mg, 0.234 mmol) in DMF (3 mL) was treated with an excess amount of NH_4OH at room temperature and gradually heated to 50 °C. After stirring for 24 h, the reaction mixture was cooled and concentrated under reduced pressure to afford compound **8** (80 mg) in 94% yield as a yellow oil, which was directly used for the next reaction.; ^1H NMR (300 MHz, CD_3OD) δ 8.41 (s, 1H), 8.14 (s, 1H), 8.01–7.99 (m, 1H), 7.71–7.65 (m, 1H), 7.24 (t, 1H, J = 8.97 Hz), 7.16 (s, 1H), 4.35–4.24 (m, 4H), 3.99 (s, 3H). MS (ESI) m/z 363 $[M + H]^+$.

*4.2.6. 4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl ((benzyloxy)carbonyl)-l-leucinate (**9**)*

Compound **9** was prepared by following the previous procedure reported in reference 15 in 32% yield as a white solid.; ^{1}H NMR (300 MHz, CD_3OD) δ 8.51 (s, 1H), 8.03 (s, 1H), 8.01 (m, 1H), 7.66–7.63 (m, 1H), 7.37–7.20 (m, 7H), 5.14 (s, 2H), 4.55 (m, 1H), 3.92 (s, 3H), 1.85 (m, 2H), 1.03 (t, 1H, J = 6.24 Hz); MS (ESI) m/z 567 $[M + H]^+$.

*4.2.7. 4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl (S)-2-(benzyloxy)-4-methylpentanoate (**10**)*

Compound **10** was prepared by following the previous procedure reported in reference 15 in 58% yield as a white solid.; ^{1}H NMR (300 MHz, CD_3OD) δ 8.54 (s, 1H), 8.17 (s, 1H), 8.02 (m, 1H), 7.70–7.65 (m, 1H), 7.41–7.22 (m, 7H), 4.55 (d, 1H, J = 11.70 Hz), 4.33 (m, 1H), 4.01 (s, 3H), 1.88 (m, 1H), 1.23 (t, 1H, J = 7.14 Hz), 0.99 (d, 3H, J = 6.42 Hz), 0.91 (d, 3H, J = 6.39 Hz); MS (ESI) m/z 524 $[M + H]^+$.

*4.2.8. 4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl l-leucinate (**11**)*

Compound **11** was prepared by following the previous procedure reported in reference 15 in 58% yield as a white solid.; ^{1}H NMR (300 MHz, DMSO) δ 9.68 (s, 2H), 9.47 (s, 1H), 8.46 (s, 1H), 8.20 (dd, 1H, J = 6.78, 2.58 Hz), 7.83 (m, 1H), 7.77 (s, 1H), 7.40 (t, 1H, J = 8.97 Hz), 7.20 (s, 1H), 3.96 (s, 3H), 3.60 (m, 1H), 1.44 (m, 1H), 1.22 (s, 2H), 0.89–0.82 (m, 6H); MS (ESI) m/z 433 $[M + H]^+$.

*4.2.9. 4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl (S)-2-hydroxy-4-methylpentanoate (**12**)*

A cooled solution of compound **10** (52.4 mg, 0.1 mmol) in CH_2Cl_2 (2 mL) at -78 °C was slowly treated with BBr_3 (1.0 M, 0.15 mL, 0.15 mmol) and gradually warmed to room temperature. After stirring for 30 min, the reaction mixture was cooled in ice-bath and then quenched with MeOH. The reaction mixture was concentrated *in vacuo* and the residue was purified by silica gel flash column chromatography to afford compound **12** (26 mg) in 60% yield as a white solid.; ^{1}H NMR (300 MHz, CD_3OD) δ 8.44 (s, 1H), 8.02 (s, 1H), 7.92 (dd, 1H, J = 6.60, 2.58 Hz), 7.57 (m, 1H), 7.20 (s, 1H), 7.16 (t, 1H, J = 8.97 Hz), 4.41 (m, 1H), 3.88 (s, 3H), 1.91 (m, 1H), 1.73–1.68 (m, 2H), 0.94 (dd, 6H, J = 6.75, 2.73 Hz); MS (ESI) m/z 434 $[M + H]^+$.

*4.2.10. 4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl (((benzyloxy)carbonyl)-l-leucyl)sulfamate (**13**)*

Compound **13** was prepared by following the previous procedure reported in reference 14 in 45% yield as a white solid.; ^{1}H NMR (300 MHz, CD_3OD) δ 8.45 (s, 1H), 8.19 (s, 1H), 7.95 (dd, 1H, J = 6.78, 2.55 Hz), 7.20 (s, 1H), 7.17–7.13 (m, 6H), 4.90 (d, 2H, J = 4.02 Hz), 4.15–4.08 (m, 1H), 3.98 (s, 3H), 1.72 (m, 1H), 1.56–1.49 (m, 2H), 0.99 (d, 6H, J = 6.57 Hz); MS (ESI) m/z 646 $[M + H]^+$.

*4.2.11. (S)-1-((((4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)sulfonyl)amino)-4-methyl-1-oxopentan-2-yl acetate (**14**)*

Compound **14** was prepared by following the previous procedure reported in reference 14 in 49% yield as a white solid.; ^{1}H NMR (300 MHz, CD_3OD) δ 8.50 (s, 1H), 8.14 (s, 1H), 8.00 (dd, 1H, J = 6.78, 2.76 Hz), 7.71–7.67 (m, 1H), 7.25 (t, 1H, J = 8.97 Hz), 7.24 (s, 1H), 4.00 (s, 3H), 2.09 (s, 3H), 1.70–1.58 (m, 3H), 0.90 (dd, 6H, J = 6.60, 4.95 Hz); MS (ESI) m/z 555 $[M + H]^+$.

*4.2.12. 4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl (l-leucyl)sulfamate (**15**)*

Compound **15** was prepared by following the previous procedure reported in reference 15 in 52% yield as a white solid.; ^{1}H NMR (300 MHz, CD_3OD) δ 8.34 (s, 1H), 7.69–7.64 (m, 3H), 7.16 (s, 1H), 7.12 (t, 1H, J = 8.61 Hz), 4.04 (s, 3H), 3.98 (dd, 1H, J = 10.44, 3.12 Hz), 1.85–1.75 (m, 1H), 1.70–1.46 (m, 2H), 0.93 (t, 6H, J = 6.57 Hz); MS (ESI) m/z 512 $[M + H]^+$.

*4.2.13. 4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl (S)-(2-hydroxy-4-methylpentanoyl)sulfamate (**16**)*

Compound **16** was prepared by following the previous procedure reported in reference 15 in 46% yield as a white solid, mp = 215–217 °C; ^{1}H NMR (400 MHz, DMSO) δ 9.60 (s, 1H), 9.46 (s, 2H), 8.46 (s, 1H), 8.20 (dd, 1H, J = 6.78, 2.58 Hz), 7.85–7.79 (m, 1H), 7.76 (s, 1H), 7.40 (t, 1H, J = 9.18 Hz), 7.20 (s, 1H), 3.96 (s, 3H), 3.15 (d, 1H, J = 4.38 Hz), 1.98 (m, 1H), 1.75–1.55 (m, 2H), 0.93 (m, 6H); ^{13}C NMR (100 MHz, DMSO) δ 169.75, 167.9, 157.3, 154.4, 148.8, 146.8, 146.4, 138.2, 121.5, 118.6, 118.3, 114.2, 112.7, 108.5, 103.0, 68.2, 56.1, 43.4, 25.28, 24.41, 22.50; MS (FAB) m/z 513 $[M + H]^+$; HRMS (FAB) m/z calcd for $C_{21}H_{22}ClFN_4O_6S$ $[M + H]^+$ 513.0933, found: 513.1021. Anal.; HPLC 97%.

*4.2.14. 2-((4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl ((benzyloxy)carbonyl)-l-leucinate (**17**)*

Compound **17** was prepared by following the previous procedure reported in reference 15 in 37% yield as a white solid.; ^{1}H NMR (300 MHz, CD_3OD) δ 8.45 (s, 1H), 8.01 (dd, 1H, J = 6.75, 2.73 Hz), 7.75 (s, 1H), 7.68 (m, 1H), 7.27–7.16 (m, 7H), 5.03 (s, 2H), 4.60–4.40 (m, 4H), 4.23 (t, 1H, J = 6.96 Hz), 3.97 (s, 3H), 1.68 (m, 1H), 1.59–1.54 (m, 2H), 0.88 (d, 3H, J = 6.39 Hz), 0.85 (d, 3H, J = 6.39 Hz); MS (ESI) m/z 611 $[M + H]^+$.

*4.2.15. 2-((4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl (S)-2-acetoxy-4-methylpentanoate (**18**)*

Compound **18** was prepared by following the previous procedure reported in reference 15 in 43% yield as a white solid.; ^{1}H NMR (300 MHz, CD_3OD) δ 8.45 (s, 1H), 8.00 (dd, 1H, J = 6.78, 2.58 Hz), 7.76 (s, 1H), 7.69–7.64 (m, 1H), 7.25 (t, 1H, J = 8.97 Hz), 7.18 (s, 1H), 5.02–4.95 (m, 2H), 4.64–4.50 (m, 2H), 4.42 (m, 1H), 4.00 (s, 3H), 2.06 (s, 3H), 1.79–1.57 (m, 3H), 0.96–0.86 (m, 6H); MS (ESI) m/z 535 $[M + H]^+$.

*4.2.16. Benzyl (S)-(1-((2-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl)amino)-4-methyl-1-oxopentan-2-yl) carbamate (**19**)*

Compound **19** was prepared by following the previous procedure reported in reference 15 in 33% yield as a white solid.; ^{1}H NMR (300 MHz, CD_3OD) δ 8.46 (s, 1H), 8.05 (dd, 1H, J = 6.78, 2.58 Hz), 7.79 (s, 1H), 7.68 (m, 1H), 7.28–7.16 (m, 7H), 5.02 (s, 2H), 4.24 (m, 2H), 4.13 (m, 1H), 3.98 (s, 3H), 3.69 (m, 2H), 1.79–1.57 (m, 3H), 0.91 (t, 6H, J = 6.39 Hz); MS (ESI) m/z 611 $[M + H]^+$.

*4.2.17. (S)-1-((2-((4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl)amino)-4-methyl-1-oxopentan-2-yl acetate (**20**)*

Compound **20** was prepared by following the previous procedure reported in reference 15 in 28% yield as a white solid.; ^{1}H NMR (300 MHz, CD_3OD) δ 8.36 (s, 1H), 7.96 (dd, 1H, J = 6.78, 2.58 Hz), 7.71 (s, 1H), 7.61 (m, 1H), 7.16 (t, 1H, J = 8.97 Hz), 7.10 (s, 1H), 4.92 (m, 1H), 4.17 (m, 2H), 3.92 (s, 3H), 3.59 (m, 2H), 2.00 (s, 3H), 1.64 (m, 1H), 1.48 (m, 2H), 0.82 (t, 6H, J = 6.42 Hz); MS (ESI) m/z 519 $[M + H]^+$.

*4.2.18. 2-((4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl l-leucinate (**21**)*

Compound **21** was prepared by following the previous procedure reported in reference 15 in 54% yield as a white solid, mp = 199–202 °C; ^{1}H NMR (400 MHz, CD_3OD) δ 8.44 (s, 1H), 8.00 (dd, 1H, J = 6.78, 2.58 Hz), 7.75 (s, 1H), 7.69–7.66 (m, 1H), 7.26 (t, 1H, J = 6.42 Hz), 7.19 (s, 1H), 4.26 (m, 2H), 4.00 (s, 3H), 3.99 (m, 2H), 3.69 (m, 1H), 1.75–1.50 (m, 3H), 0.92 (t, 6H, J = 6.21 Hz);); ^{13}C NMR (100 MHz, DMSO) δ 169.75, 156.57, 156.00, 154.32, 153.91, 152.68,

148.05, 147.01, 123.37, 122.23, 118.80, 116.56, 108.68, 107.37, 102.55, 66.70, 62.30, 53.81, 43.40, 38.54, 38.16, 25.28, 24.41, 22.50; MS (FAB) *m/z* 477 $[M + H]^+$; HRMS (FAB) *m/z* calcd for $C_{23}H_{26}ClFN_4O_4$ $[M + H]^+$ 477.1627, found: 477.1716. Anal.; HPLC 95%.

4.2.19. 2-((4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl (S)-2-hydroxy-4-methylpentanoate (22)

Compound **22** was prepared by following the previous procedure reported in reference 15 in 54% yield as a white solid, mp = 196–203 °C; ^{1}H NMR (400 MHz, DMSO) δ 11.05 (s, 1H), 8.81 (s, 1H), 8.20 (s, 1H), 8.03 (m, 1H), 7.73 (m, 1H), 7.65 (t, 1H, J = 8.79 Hz), 7.31 (s, 1H), 4.24 (m, 2H), 4.00 (s, 3H), 3.84 (m, 2H), 2.18 (m, 1H), 1.76 (m, 2H), 0.87 (m, 6H); ^{13}C NMR (100 MHz, DMSO) δ 169.75, 156.57, 156.00, 154.32, 153.91, 152.68, 148.05, 147.01, 123.37, 122.23, 118.80, 116.56, 108.68, 107.37, 102.55, 67.90, 55.81, 43.40, 38.54, 38.16, 25.28, 24.41, 22.50; MS (FAB) *m/z* 478 $[M + H]^+$; HRMS (FAB) *m/z* calcd for $C_{23}H_{25}ClFN_3O_5$ $[M + H]^+$ 478.1467, found: 478.1556. Anal.; HPLC 96%.

4.2.20. (S)-2-Amino-N-(2-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl)-4-methylpentanamide (23)

Compound **23** was prepared by following the previous procedure reported in reference 15 in 55% yield as a white solid, mp = 198–202 °C; ^{1}H NMR (400 MHz, DMSO) δ 8.49 (s, 1H), 8.12 (s, 1H), 7.99 (dd, 1H, J = 6.78, 2.58 Hz), 7.75 (s, 1H), 7.71–7.66 (m, 1H), 7.25 (t, 1H, J = 8.97 Hz), 7.16 (s, 1H), 4.25 (m, 2H), 3.98 (s, 3H), 3.80 (m, 1H), 3.73 (m, 2H), 1.75–1.58 (m, 3H), 0.90 (dd, 6H, J = 6.21, 5.31 Hz); ^{13}C NMR (175 MHz, DMSO) δ 169.75, 156.57, 156.00, 154.32, 153.91, 152.68, 148.05, 147.01, 123.37, 122.23, 118.80, 116.56, 108.68, 107.37, 102.55, 67.66, 55.81, 54.86, 38.54, 38.16, 25.28, 24.41, 22.50; MS (FAB) *m/z* 476 $[M + H]^+$; HRMS (FAB) *m/z* calcd for $C_{23}H_{27}ClFN_5O_3$ $[M + H]^+$ 476.1786, found: 476.1876. Anal.; HPLC 95%.

4.2.21. (S)-N-(2-((4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl)-2-hydroxy-4-methylpentanamide (24)

Compound **24** was prepared by following the previous procedure reported in reference 15 in 57% yield as a white solid.; ^{1}H NMR (300 MHz, CD_3OD) δ 8.36 (s, 1H), 7.94 (d, J = 6.96 Hz), 7.73 (s, 1H), 7.60 (m, 1H), 7.15 (t, 1H, J = 8.97 Hz), 7.09 (s, 1H), 4.18 (t, 2H, J = 5.49 Hz), 3.97 (m, 1H), 3.92 (s, 3H), 3.62 (m, 2H), 1.75 (m, 1H), 1.37–1.45 (m, 2H), 0.82 (dd, 6H, J = 6.68, 4.05 Hz); MS (ESI) *m/z* 477 $[M + H]^+$.

4.2.22. 2-((4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl (((benzyloxy)carbonyl)-l-leucyl)sulfamate (25)

Compound **25** was prepared by following the previous procedure reported in reference 14 in 46% yield as a white solid.; ^{1}H NMR (300 MHz, CD_3OD) δ 8.43 (s, 1H), 8.01 (dd, 1H, J = 6.78, 2.58 Hz), 7.91 (s, 1H), 7.67 (m, 1H), 7.20–7.11 (m, 7H), 4.94 (s, 2H), 4.41–4.37 (m, 4H), 4.08 (m, 1H), 3.96 (s, 3H), 1.75–1.45 (m, 3H), 0.86 (m, 6H); MS (ESI) *m/z* 690 $[M + H]^+$.

4.2.23. (S)-1-(((2-((4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethoxy)sulfonyl)amino)-4-methyl-1-oxopentan-2-yl acetate (26)

Compound **26** was prepared by following the previous procedure reported in reference 14 in 58% yield as a white solid.; ^{1}H NMR (300 MHz, CD_3OD) δ 8.41 (s, 1H), 8.04 (dd, 1H, J = 6.78, 2.76 Hz), 7.92 (s, 1H), 7.76–7.71 (m, 1H), 7.17 (t, 1H, J = 8.97 Hz), 7.10 (s, 1H), 4.83–4.79 (m, 1H), 4.44 (m, 4H), 3.98 (s, 3H), 1.74–1.55 (m, 3H), 0.87 (dd, 6H, J = 9.54, 6.60 Hz); MS (ESI) *m/z* 599 $[M + H]^+$.

4.2.24. 2-((4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl (l-leucyl)sulfamate (27)

Compound **27** was prepared by following the previous procedure reported in reference 15 in 65% yield as a white solid.; ^{1}H NMR (300 MHz, CD_3OD) δ 8.44 (s, 1H), 8.06 (dd, 1H, J = 6.77, 2.76 Hz), 7.89 (s, 1H), 7.73 (m, 1H), 7.22 (t, 1H J = 8.97 Hz), 7.14 (s, 1H), 4.48 (m, 4H), 3.98 (s, 3H), 3.60 (m, 1H), 1.56–1.75 (m, 3H), 0.91 (m, 6H); MS (ESI) *m/z* 556 $[M + H]^+$.

4.2.25. 2-((4-((3-Chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl (S)-(2-hydroxy-4-methylpentanoyl)sulfamate (28)

Compound **28** was prepared by following the previous procedure reported in reference 15 in 77% yield as a white solid.; ^{1}H NMR (300 MHz, CD_3OD) δ 8.43 (s, 1H), 8.06 (dd, 1H, J = 6.78, 2.76 Hz), 7.90 (s, 1H), 7.70 (m, 1H), 7.20 (t, 1H, J = 9.15 Hz), 7.12 (s, 1H), 4.80 (m, 1H), 4.47 (m, 4H), 3.98 (s, 3H), 1.80 (m, 1H), 1.55–1.42 (m, 2H), 0.86 (dd, 6H, J = 6.60, 1.65 Hz); MS (ESI) *m/z* 557 $[M + H]^+$.

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