Tranilast-tyrosine hybrid molecule exhibits dual activity: suppression of epithelial-mesenchymal transition and induction of cytotoxicity in cancer cells

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Abstract
The anti-allergic drug tranilast (TNL) reportedly exhibits inhibitory activity against epithelial-mesenchymal transition (EMT) at high concentrations. Herein, we synthesized a new hybrid molecule, tyrosine-conjugated TNL having a propyl linker (TNL-T), which is expected to increase anti-EMT activity by enhancing intracellular delivery through an amino acid transporter expressed in cancer cells. The anti-EMT activity of TNL-T was similar to that of TNL in the lung carcinoma cell line A549. Unlike TNL, TNL-T could induce dose-dependent cytotoxicity in A549 cells; this cytotoxicity was also observed in the glioblastoma cell lines U251 and U87. However, neither tyrosine nor TNL exhibited such cytotoxicity, demonstrating the importance of the TNL-T structure. The derivatization study showed that a derivative with an ethyl linker has the similar functions, and that of a pentyl linker was inactive. TNL-T, which exhibited dual activity by suppressing EMT and cancer cell proliferation, could aid in the development of new anticancer drugs.

Keywords Tranilast · Tyrosine · Hybrid molecule · Anti-epithelial-mesenchymal transition · Cytotoxicity · Cancer cells

Introduction
The human cell membrane can regulate the inbound/outbound transport of molecules. As certain drugs/biologically active compounds fail to penetrate this membrane easily, precise strategies are often required to achieve membrane permeability. For example, to improve the permeability of highly charged molecules such as phosphoinositides, produgs have been developed by introducing neutral substituents that allow penetration across the lipophilic membrane and removal by esterase from within the cell [1–3].

The use of membrane transporters that recognize extracellular biomolecules and convey them across the membrane is another specific strategy for intracellular drug delivery. L-type amino acid transporter 1 (LAT1) [4] is an example of such transporters. LAT1 transports large neutral amino acids, such as tyrosine, and is especially expressed in human cancer cells, which demand large amounts of amino acids for cell proliferation. Moreover, various amino acid-conjugated anticancer drugs have been developed for targeted and efficient delivery into cancer cells [5, 6]. Indeed, L-phenylalanine-conjugated mechlorethamine,
termed melphalan, has been used in clinics to treat cancer for a considerable time [7].

The anti-atopic activity of tranilast (TNL) (Fig. 1) was first reported in 1976 [8]. Since then, this drug has been used to treat allergic diseases, and various effects, including those other than its anti-allergic activity, have been reported [9]. Although the direct target molecules have remained elusive, TNL was found to directly target NOD-like receptor family pyrin domain containing 3 (NLRP3) and inhibit inflammasome activation [10]. One notable TNL-mediated function is the inhibition of epithelial-mesenchymal transition (EMT) followed by cancer metastasis [11–13], which is closely related to cancer mortality [14, 15].

However, high concentrations of TNL (≥100 μM) are required to induce sufficient anti-EMT activity [13, 16, 17]. We hypothesized that introducing a tyrosine moiety would increase the intracellular content of the compound, improving anti-EMT activity. Herein, we report the synthesis of a new hybrid molecule, tyrosine-conjugated TNL, and its unexpected dual activity against EMT and cell proliferation.

**Results**

**Synthesis of a tranilast-tyrosine hybrid molecule (TNL-T)**

Typically, amino acids are directly linked to specific drugs to synthesize amino acid-conjugated drugs [6]. In the present study, we designed a hybrid molecule, TNL-T (Fig. 1), by conjugating tyrosine to tranilast via a short linker of three carbons. Introducing the linker may facilitate the binding of tyrosine and TNL to LAT1 and a target protein of TNL, respectively.

TNL-T was synthesized as shown in Scheme 1. Boc-tyrosine tert-butyl ester 1 and 1,3-dibromopropane were heated under reflux in acetone in the presence of K2CO3 to generate compound 2 (69% yield). TNL and compound 2 were dissolved in acetonitrile and heated to 60 °C in the presence of K2CO3 to obtain compound 3 (28% yield). Compound 3 was deprotected with trifluoroacetic acid (TFA) in CH2Cl2 to generate TNL-T (47% yield).

To improve the solubility of TNL-T in dimethyl sulfoxide (DMSO) and cell culture medium, the hydrochloride salt of TNL (TNL-T·HCl) was obtained by treating TNL-T with 6N HCl in MeOH (quantitative yield). TNL-T·HCl was used in further experiments to examine the biological activity of TNL-T.

**Biological activity of TNL-T in lung cancer cells**

To analyze the anti-EMT activity of TNL and TNL-T, we used the lung carcinoma cell line A549. First, we examined the cytotoxicity of TNL and TNL-T in A549 cells. After normal seeding, the cells were incubated, and compounds were added to the culture medium at various concentrations (10–300 μM). The cells were further incubated for 3 days, and the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed. As shown in Fig. 2a, TNL enhanced proliferation in a dose-dependent manner from 10 μM to 200 μM. In contrast, TNL-T exhibited dose-dependent cytotoxicity in the range of 10–100 μM, which subsequently plateaued. The cells were then seeded at a lower concentration, and the growth curve was observed in the presence of treatment compounds; the results are shown in Fig. 2b. The TNL and TNL-T activities, shown in Fig. 2a, were clearly
observed in Fig. 2b. Notably, the effect of TNL (50–300 μM) on cell proliferation was observed after 2 days, exhibiting cytotoxicity after 1 day. TNL (10 μM) showed a weak inhibitory activity. TNL-T suppressed proliferation in a dose-dependent manner, reducing ~50% of living cells at concentrations of ≥100 μM on day 3. Given the modest cytotoxicity observed in these experiments, we next examined the anti-EMT effect in A549 cells.
Transforming growth factor (TGF)-β-stimulated A549 cells were incubated with TNL or TNL-T (10, 100, or 300 μM) for 3 days, followed by cell lysis. The total protein content in the cell lysate was normalized, and levels of E-cadherin, an epithelial marker protein, and Zeb1, a mesenchymal marker protein, were analyzed by immunoblotting. As shown in Fig. 3a, cell stimulation with TGF-β decreased levels of E-cadherin and increased those of Zeb1. TNL dose-dependently suppressed these changes in E-cadherin and Zeb1 levels, and TNL-T also exhibited a similar effect. EMT is known to induce cell migration, and scratch assays were performed using TGF-β-stimulated A549 cells incubated with the test compounds for 1 day. As shown in Fig. 3b, both TNL and TNL-T suppressed cell migration, and a concentration of 100 μM inhibited migration by approximately 50%. Notably, a previous study has also shown that TNL can induce anti-EMT and anti-cell migration activities [18].

As biological activities of TNT-L in A549 cells, the anti-EMT activity of TNL was not improved. However, TNL-T demonstrated dose-dependent cytotoxicity, in contrast to TNL, which enhanced cell proliferation.

### Cytotoxic activity of TNL-T

Next, the cytotoxicity of TNL-T was analyzed using the glioblastoma cell lines U251 and U87. Growth curves were examined in the presence of TNL and TNL-T by performing the same experiment conducted using A549 cells (Fig. 2b). As shown in Fig. 4a, all examined TNL concentrations enhanced U251 cell proliferation. In U87 cells, 10 μM TNL suppressed proliferation, while ≥50 μM TNL failed to demonstrate clear activity (Fig. 4b). In contrast, TNL-T suppressed the proliferation of these cell lines in a dose-dependent manner; this TNL-T-mediated effect was observed in both lung carcinoma and glioblastoma cells. The mechanism of cytotoxicity is currently under investigation.

We next performed the same experiment shown in Fig. 2a to examine the cytotoxicity of tyrosine, a component of TNL-T. As shown in Fig. 5a, tyrosine (10–300 μM) did not exhibit any clear activity. The proliferation enhancement/suppression activities mediated by these compounds in U251/U87 were confirmed in this experiment (Fig. 5b).

These results suggested that both TNL and tyrosine are crucial to induce TNL-T-mediated cytotoxicity.

### TNT-T derivatization study

Finally, effect of the length of a linker in TNL-T was examined. We designed derivatives TNL-T2 and TNL-T5 with shorter ethyl and longer pentyl linkers, respectively (Fig. 6a). The hydrochloride salts of the compounds (TNL-T2·HCl and TNL-T5·HCl) were prepared according to the synthetic route of TNL-T·HCl (Scheme 1) using bromides 2b and 2c. The hydrochloride salts were used for the subsequent experiments.

Cytotoxicity and anti-cell migration activity of TNL-T2 and TNL-T5 were examined by the same method as that shown in Figs. 2a and 3b. As shown in Fig. 6b, TNL-T2 showed cytotoxicity similar to TNL-T (Fig. 2a), whereas TNL-T5 did not show the activity. Furthermore, TNL-T2 showed anti-cell migration activity (Fig. 6c) like TNL-T (Fig. 3b), in contrast to no activity of TNL-T5 (Fig. 6c). Replacement with a shorter linker of two carbons does not affect on the dual activity of TNL-T. Notably, more hydrophobic TNL-T5 were not sufficiently soluble in culture medium, and this would be the cause of inactivity in these experiments.

### Discussion

In the present study, we synthesized a new hybrid molecule, TNL-T, intending to improve the anti-EMT activity of TNL by increasing the cellular uptake through LAT1 transporter.
We found that TNL-T and TNL exhibited similar anti-EMT activities in lung carcinoma cells. This suggests a possibility that TNL-T transported inside the cell mostly remained intact, not cleaved by intracellular esterase. Supposing that the intrinsic anti-EMT activity of TNL-T is weaker than TNL, larger amount of TNL-T transported into the cell may show almost the same activity of TNL lesser amount penetrated inside the cell.

We unexpectedly found that TNL-T showed dose-dependent cytotoxicity in lung carcinoma and glioblastoma cells. Among lung carcinoma A549 and glioblastoma cells U251 and U87, U251 showed the highest sensitivity to 10 μM of TNL-T at 3 days in cell growth experiment (Figs. 2b and 4a, b). There are reports that U251 expresses much higher LAT1 compared with U87 [19] and U87 and A549 express similar degree of LAT1 [20]. Thus, the effect of TNL-T against U251, U87 and A549 cells seemed dependent on the LAT1 expression. On the other hand, under certain conditions, TNL exhibited cytotoxicity (Figs. 2b and 4b); however, the ability to enhance cell proliferation appears to overcome its cytotoxicity. TNL-T did not enhance cell proliferation and showed dose-dependent cytotoxicity. These different activities shown in TNL-T and TNL would be caused by difference of structural and/or amounts inside the cell.

To exert dose-dependent cytotoxicity, both the TNL part and the tyrosine part of TNL-T are required. In this study, we first employed a propyl linker between the TNL and the tyrosine parts. Our ethyl and pentyl derivatization study showed importance of linker length. Further derivatization would alter the amount of intracellular uptake, the sensitivity to esterase, the cytotoxicity, and the anti-EMT activity. It remains unclear how TNL-T, or even TNL, exerted anti-EMT and anti-cell proliferation activity. Further, we need evidences to show that cellular uptake of TNL-T is LAT1-dependent and that TNL-T is not cleaved by intracellular esterase. This should lead to rational design and improvement of TNL-T.

Conclusions

A new hybrid molecule TNL-tyrosine named TNL-T was synthesized and its activities were elucidated. Despite EMT activity was not increased compared to TNL, its unique activity, dose-dependent cytotoxicity was disclosed. Dual activities that involve suppression of cell proliferation and EMT can be beneficial for reducing the number of cancer cells and inhibiting metastasis. Thus, TNL-T could be a lead for new anticancer drugs.
Material and methods

General procedure pertaining to synthesis

The reagents used in the synthesis were purchased from Wako Pure Chemical Industries (Osaka, Japan), Tokyo Chemical Industry (Tokyo, Japan), and Nacalai Tesque (Kyoto, Japan). Silica gel 60N (spherical, neutral) (Kanto Chemical, Tokyo, Japan) was used for column chromatography. Silica gel 60F254 (Merck, Darmstadt, Germany) was used for thin layer chromatography. The melting point (mp) was measured using Yanaco (Kyoto, Japan) MP-S3. The NMR spectrum was measured by JEOL (Tokyo, Japan) JNM-AL300 and Bruker (Billerica, MA, USA) AVANCE 600, and the chemical shift value was expressed in ppm using the NMR measurement solvent as a reference substance. The J value was expressed in Hz. The IR spectrum was measured by JASCO (Tokyo, Japan) FT/IR-410. The high-resolution mass spectrum (HRMS) was measured by the JOEL JMS-DX-303-HF mass spectrometer.

Synthesis of TNL

Tranilast (TNL) was synthesized according to the industrial synthesis [21].

Synthesis of TNL-T ∙ HCl

\[ \text{N-tert-Butoxycarbonyl-O-3-bromopropyltyrosine tert-butyl ester (2): K}_2\text{CO}_3 \ (829 \text{ mg}, \ 6.00 \text{ mmol}) \] was heated to
dehydration under reduced pressure, cooled to room temperature, and acetone (4.0 mL) was added. Boc-Tyrosine tert-butyl ester 1 [22] (674 mg, 2.00 mmol) and 1,3-dibromopropane (0.40 mL, 3.9 mmol) were added, and the mixture was heated under reflux for 15 h. The solution was neutralized with 5% succinic acid and purified water (20 mL) was added. Acetone was distilled off and the residue was purified by silica gel column chromatography (Hexane:AcOEt = 3:1) to give a pale yellow millet jelly-like compound 2 (69% yield). $^1$H NMR (CDCl$_3$) $\delta$ 1.41

Fig. 6 Cytotoxicity and anti-EMT activities of TNL-T derivatives in A549 cells. a Structures of the derivatives, TNL-T2 and TNL-T5, and their synthetic intermediates. b Cell viability. This experiment is similar to that shown in Fig. 2a. c Cell migration. This experiment is similar to that shown in Fig. 3b. $^*$P < 0.05, $^{**}$P < 0.01, $^{***}$P < 0.001 compared with the sample without drugs, n.s. not significant compared with the sample without drugs. TNL-T2 tranilast-tyrosine conjugate with a linker of two carbons, TNL-T5 tranilast-tyrosine conjugate with a linker of five carbons.
N-tert-Butyloxycarbonyl-O-[3-[2-(3,4-dimethoxy-ycinnamoylamino)benzoyloxy]propyl]tyrosine tert-butyl ester (3): K$_2$CO$_3$ (91.1 mg, 0.659 mmol) was heated to dehydration under reduced pressure, cooled to room temperature, and then dry acetonitrile (4.0 mL) was added. Tranilast (TNL) (216 mg, 0.660 mmol) and compound 2 (304 mg, 0.663 mmol) were suspended therein and stirred at 60 °C for 14 h. The solution was cooled to room temperature, neutralized with 5% succinic acid, and the acetonitrile was distilled off. To the residue was added 5% succinic acid (20 mL) and the solution was extracted with CH$_2$Cl$_2$ (10 mL x3), washed with brine, and dried over MgSO$_4$. The organic layer was concentrated under reduced pressure and the residue was purified by silica gel chromatography (Hexane:AcOEt = 2:1) to give a white solid 3 (28% yield). mp: 61–63 °C. $^1$H NMR (CDCl$_3$) δ 1.409 (s, 9H), 1.412 (s, 9H), 2.27–2.31 (m, 2H), 2.97–3.00 (m, 2H), 3.93 (s, 3H), 3.96 (s, 3H), 4.12–4.14 (t, J = 6.0, 2H), 4.55–4.57 (t, J = 6.3, 2H), 4.95–4.96 (d, J = 7.9, 1H), 6.47–6.50 (d, J = 15.5, 2H), 6.82–6.84 (dd, J = 6.8, 1.9, 1.7, 2H), 6.88–6.89 (dd, J = 8.3, 1H), 7.07–7.11 (m, 4H), 7.15–7.17 (dd, J = 8.3, 1.9, 1H), 7.57–7.60 (dt, J = 7.9, 1.6, 1.3, 1H), 7.69–7.71 (d, J = 15.5, 1H), 8.05–8.07 (dd, J = 8.0, 1.6, 1H), 8.87–8.89 (dd, J = 8.5, 0.9, 1H). $^{13}$C NMR (CDCl$_3$) δ 28.0, 28.3, 28.7, 37.6, 55.0, 56.0, 56.0, 62.4, 64.3, 79.6, 82.0, 109.8, 111.1, 114.4, 114.9, 119.7, 120.7, 122.4, 122.6, 127.1, 128.8, 130.6, 130.8, 134.8, 142.1, 142.3, 149.3, 151.0, 155.1, 157.7, 164.8, 168.5, 171.0. IR (KBr) 3374, 2977, 2923, 1716, 1612, 1365, 1245, 1160, 1037, 922, 844.

Synthesis of TNL-T2·HCl

N-tert-Butyloxycarbonyl-O-2-bromoethyltyrosine tert-butyl ester (2b): According to the synthetic method of 2, 2b was synthesized from 1 $^{[22]}$, and 2b was used for the next step without purification.

N-tert-Butyloxycarbonyl-O-[2-[3-(4-dimethoxyycinnamoylamino)benzoyloxy]ethyl]tyrosine tert-butyl ester: According to the synthetic method of 3, this compound was synthesized from 2b. This compound was obtained as a white solid in 30% yield from 1 (2 steps). mp: 92–94 °C. $^1$H NMR (CDCl$_3$) δ 1.42 (d, J = 1.3, 18H), 2.96–3.04 (m, 2H), 3.93 (s, 3H), 3.96 (s, 3H), 4.31–4.33 (m, 2H), 4.40–4.41 (m, 1H), 4.68–4.70 (m, 2H), 6.49 (d, J = 15.5, 1H), 6.86–6.89 (m, 3H), 7.08–7.11 (m, 4H), 7.16 (dd, J = 8.5, 2.0, 1H), 7.59 (dd, J = 8.7, 7.3, 1.6, 1H), 7.71 (d, J = 15.5, 1H), 8.09 (dd, J = 8.0, 1.5, 1H), 8.88 (dd, J = 8.5, 1.0, 1H), 11.22 (s, 1H). $^{13}$C NMR (CDCl$_3$) δ 28.0, 28.3, 56.0, 56.0, 58.9, 63.7, 65.6, 82.0, 109.8, 111.1, 114.5, 114.7, 116.9, 119.7, 120.7, 122.5, 122.6, 127.7, 128.9, 130.7, 131.1, 135.0, 142.1, 142.4, 149.3, 151.0, 164.8, 168.4, 175.1, 175.1. HRMS (FAB) m/z Calcd. for C$_{38}$H$_{40}$O$_{25}$N$_2$ [M + H]$^+$ 549.2231. Found: 549.2245.

Synthesis of TNL-T2·HCl

N-tert-Butyloxycarbonyl-O-2-bromoethyltyrosine tert-butyl ester (2b): According to the synthetic method of 2, 2b was synthesized from 1 $^{[22]}$, and 2b was used for the next step without purification.

N-tert-Butyloxycarbonyl-O-[2-[3-(4-dimethoxyycinnamoylamino)benzoyloxy]ethyl]tyrosine tert-butyl ester: According to the synthetic method of 3, this compound was synthesized from 2b. This compound was obtained as a white solid in 30% yield from 1 (2 steps). mp: 92–94 °C. $^1$H NMR (CDCl$_3$) δ 1.42 (d, J = 1.3, 18H), 2.96–3.04 (m, 2H), 3.93 (s, 3H), 3.96 (s, 3H), 4.31–4.33 (m, 2H), 4.40–4.41 (m, 1H), 4.68–4.70 (m, 2H), 6.49 (d, J = 15.5, 1H), 6.86–6.89 (m, 3H), 7.08–7.11 (m, 4H), 7.16 (dd, J = 8.5, 2.0, 1H), 7.59 (dd, J = 8.7, 7.3, 1.6, 1H), 7.71 (d, J = 15.5, 1H), 8.09 (dd, J = 8.0, 1.5, 1H), 8.88 (dd, J = 8.5, 1.0, 1H), 11.22 (s, 1H). $^{13}$C NMR (CDCl$_3$) δ 28.0, 28.3, 56.0, 56.0, 58.9, 63.7, 65.6, 82.0, 109.8, 111.1, 114.5, 114.7, 116.9, 119.7, 120.7, 122.5, 122.6, 127.7, 128.9, 130.7, 131.1, 135.0, 142.1, 142.4, 149.3, 151.0, 164.8, 168.4, 175.1, 175.1. HRMS (FAB) m/z Calcd. for C$_{38}$H$_{40}$O$_{25}$N$_2$ [M + H]$^+$ 691.3231. Found: 691.3255.

O-[2-[3-(4-dimethoxyycinnamoylamino)benzoyloxy]ethyl]tyrosine hydrochloride (TNL-T2·HCl): According to the synthetic method of TNL-T2, TNL-T2 was synthesized from the above compound. TNL-T2 (0.05 mmol) was then completely dissolved in dioxane (2 mL), and 4 M HCl/dioxane (0.2 mL) was added dropwise. The mixture was stirred for 4 h, and concentrated under reduced pressure to give a precipitate that was collected and dried to give TNL-T2·HCl as a pale-yellow solid in quantitative yield. mp: 192–194 °C. $^1$H NMR (DMSO) δ 3.03–3.06 (m, 2H), 3.81 (s, 3H), 3.83 (s, 3H), 4.05–4.08 (m, 1H), 4.34 (t, J = 6.4, 2H), 4.62 (t, J = 6.4, 2H), 6.82 (d, J = 15.6, 1H), 6.91 (d, J = 8.7, 2H), 7.00 (d, J = 8.4, 1H), 7.15 (d, J = 8.6, 2H).
obtained as a white solid in 19% yield from 

H NMR (CDCl3) δ 1.41 (s, 9H), 1.42 (s, 9H), 1.65–1.69 (m, 2H), 1.85–1.92 (m, 4H), 2.98–3.01 (m, 2H), 3.93 (s, 3H), 3.96 (s, 3H), 3.97 (t, J = 6.4, 2H), 4.33–4.45 (m, 2H), 4.96 (d, J = 8.0, 1H), 6.49 (d, J = 15.5, 1H), 6.80–6.82 (m, 2H), 6.88 (d, J = 8.3, 1H), 7.03–7.12 (m, 4H), 7.16 (dd, J = 8.4, 1.8, 1H), 7.58 (ddd, J = 8.6, 7.3, 1.6, 1H), 7.70 (d, J = 15.5, 1H), 8.07 (dd, J = 8.0, 1.6, 1H), 8.88 (dd, J = 8.5, 1.0, 1H), 11.33 (s, 1H).

13C NMR (CDCl3) δ 22.8, 28.0, 28.3, 28.4, 29.0, 31.6, 56.0, 56.1, 65.3, 67.6, 81.9, 109.8, 111.1, 114.4, 115.1, 119.7, 120.7, 122.4, 122.6, 127.7, 130.5, 130.8, 134.7, 142.1, 142.3, 145.2, 149.3, 151.0, 155.1, 164.8, 168.6, 171.0. HRMS (FAB) m/z Calcd. for C41H53N2O10 [M + H]+ 733.3700. Found: 733.3761.

O-(5-[2-(3,4-dimethoxybicyclohexyl)benzoyloxy] penty]tyrosine hydrochloride (TNL-T5-HCl): According to the synthetic method of TNL-T, TNL-T5 was synthesized from the above compound. TNL-T5 was converted to TNL-T5-HCl (a pale-yellow solid) in quantitative yield, according to the same method of TNL-T2-HCl. mp: 137–139 °C. 1H NMR (DMSO) δ 1.48–1.62 (m, 2H), 1.77 (qd, J = 14.0, 6.5 Hz, 4H), 3.00–3.09 (m, 2H), 3.80 (s, 3H), 3.83 (s, 3H), 3.93 (t, J = 6.4, 2H), 3.96 (d, J = 6.3, 1H), 4.33 (t, J = 6.5, 2H), 6.80–6.89 (m, 3H), 7.00 (d, J = 8.4, 1H), 7.15 (d, J = 8.7, 2H), 7.18–7.28 (m, 2H), 7.36 (d, J = 2.0, 1H), 7.56 (t, J = 13.4, 1H), 7.61–7.68 (m, 1H), 7.94 (dt, J = 25.8, 12.9, 1H), 8.33 (brs, 2H), 8.42 (dd, J = 8.4, 0.9, 1H). 13C NMR (DMSO) δ 22.2, 27.8, 28.3, 34.8, 53.4, 55.6, 55.6, 65.0, 67.2, 110.3, 111.6, 114.4, 114.9, 117.9, 121.3, 122.6, 123.1, 126.6, 127.3, 130.5, 130.6, 133.8, 140.0, 141.6, 149.0, 150.7, 157.8, 164.2, 167.2, 170.2. HRMS (FAB) m/z Calcd. for C32H37O8N2 [M + H]+ 577.2550. Found: 577.2570.

Synthesis of TNL-T5

N-tert-Butoxycarbonyl-O-5-bromopentyltyrosine tert-butyl ester (2c): According to the synthetic method of 2, 2c was synthesized from 1 [22], and 2c was used for the next step without purification.

N-tert-Butoxycarbonyl-O-5-[2-(3,4-dimethoxybicyclohexyl)benzoyloxy] penty]tyrosine tert-butyl ester: According to the synthetic method of 3, this compound was synthesized from 2c. This compound was obtained as a white solid in 19% yield from 1 (2 steps). mp: 58–60 °C. 1H NMR (CDCl3) δ 1.41 (s, 9H), 1.42 (s, 9H), 1.65–1.69 (m, 2H), 1.85–1.92 (m, 4H), 2.98–3.01 (m, 2H), 3.93 (s, 3H), 3.96 (s, 3H), 3.97 (t, J = 6.4, 2H), 4.33–4.45 (m, 2H), 4.96 (d, J = 8.0, 1H), 6.49 (d, J = 15.5, 1H), 6.80–6.82 (m, 2H), 6.88 (d, J = 8.3, 1H), 7.03–7.12 (m, 4H), 7.16 (dd, J = 8.4, 1.8, 1H), 7.58 (ddd, J = 8.6, 7.3, 1.6, 1H), 7.70 (d, J = 15.5, 1H), 8.07 (dd, J = 8.0, 1.6, 1H), 8.88 (dd, J = 8.5, 1.0, 1H), 11.33 (s, 1H). 13C NMR (CDCl3) δ 22.8, 28.0, 28.3, 28.4, 29.0, 31.6, 56.0, 56.1, 65.3, 67.6, 81.9, 109.8, 111.1, 114.4, 115.1, 119.7, 120.7, 122.4, 122.6, 127.7, 130.5, 130.8, 134.7, 142.1, 142.3, 145.2, 149.3, 151.0, 155.1, 164.8, 168.6, 171.0. HRMS (FAB) m/z Calcd. for C41H53N2O10 [M + H]+ 733.3700. Found: 733.3761.

Solution of compounds

Each test compound was dissolved in DMSO (Wako Pure Chemical Industries), and the solution was added to the culture medium at a 1/100 volume.

Cells

The human lung carcinoma cell line A549 (provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT/AMED, Japan [RCB0098]) and the human glioblastoma cell lines U251 and U87 were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F12, supplemented with 5% heat-inactivated fetal bovine serum.

MTT assay

Cells were seeded in a 24-well plate (for time-course experiment: 0.2 × 10^5 cells/500 μL/well; the other: 1.0 × 10^5 cells/500 μL/well) and incubated for 1 day. The test compounds were added to the cell culture medium, and cells were incubated for the designated time. MTT (DOJINDO Laboratories, Kumamoto, Japan) solution (0.25 mg/mL) was then added, and the MTT assay was performed as previously described [23].

In vitro scratch assay

Cells were seeded in a 24-well plate (1.0 × 10^5 cells/500 μL/well) and incubated for 1 day. A scratch assay was performed to evaluate cell migration in the presence of the test compounds and TGF-β (10 ng/mL) (R&D Systems Minneapolis, MN, USA), as previously described [24].

Immunoblot analysis

Cells were seeded in a 6-well plate (0.5 × 10^5 cells/2 mL/well) and incubated for 3 days. Then, test compounds were added to the cell culture medium, and cells were incubated for another 3 days. Subsequently, cells were lysed with RIPA buffer (50 mM Tris-HCl [pH 7.8], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1% protease inhibitor cocktail [Nacalai Tesque], 1% phosphatase inhibitor cocktail [Nacalai Tesque]), and the protein concentration of the lysate was determined using BCA protein assay kit (ThermoFisher Scientific, Waltham, MA, USA). Gel electrophoresis and immunoblotting were performed using 10 μg total protein per gel. E-cadherin antibody (H-108) (Santa Cruz Biotechnology, Dallas, TX, USA), zeb1 (D80D3) rabbit mAb (Cell Signaling Technology, Danvers, MA, USA), or anti-GAPDH antibody (0411) (Santa Cruz Biotechnology) were used.
Immunoreactivity was detected by chemiluminescence using ImmunoStar LD (Fujifilm Wako, Osaka, Japan).

**Data availability**

NMR spectra of TNL-T, TNL-T2 and TNL-T5 are depicted in the Supporting Information.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare no competing interests.

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