Tranilast-Tyrosine Hybrid Molecule Exhibits Dual Activity: Suppression of Epithelial-Mesenchymal Transition and Induction of Cytotoxicity in Cancer Cells

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Research Article

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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, tranilast-tyrosine (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 cytotoxicity, demonstrating the importance of the TNL-T structure. TNL-T, which exhibited dual activity by suppressing EMT and cancer cell proliferation, could aid in the development of new anticancer drugs.

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, prodrugs 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) 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 mM) 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 And Discussion

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. 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 K_2CO_3 to generate compound **2** (69% yield). TNL and compound **2** were dissolved in acetonitrile and heated to 60°C in the presence of K_2CO_3 to obtain compound **3** (28% yield). Compound **3** was deprotected with trifluoroacetic acid (TFA) in CH_2Cl_2 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 mM). 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 mM to 200 mM. In contrast, TNL-T exhibited dose-dependent cytotoxicity in the range of 10–100 mM, 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 mM) on cell proliferation was observed after 2 days, exhibiting cytotoxicity after 1 day. TNL (10 mM) showed a weak inhibitory activity. TNL-T suppressed proliferation in a dose-dependent manner, reducing approximately 50% of living cells at concentrations of ≥ 100 mM 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 mM) 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 mM 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 mM TNL suppressed proliferation, while ≥ 50 mM 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.

Finally, we 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 mM) 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.

Conclusions

In the present study, we synthesized a new hybrid molecule, TNL-tyrosine, and aimed to increase the amount of TNL entering the cell to improve the anti-EMT activity of TNL. The results showed that TNL-T and TNL exhibited almost similar anti-EMT activities in lung carcinoma cells. It remains unclear whether intracellular drug delivery had improved. However, TNL-T demonstrated dose-dependent cytotoxicity in lung carcinoma and glioblastoma cells. Under certain conditions, TNL exhibited cytotoxicity (Fig. 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. It should be noted that the entire TNL-T molecule was required to mediate these effects.

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 Tranilast (Tnl)

TNL was synthesized according to the industrial synthesis [19].

Synthesis Of Tranilast-tyrosine (Tnl-t)

N-*tert*-Butoxycarbonyl-*O*-3-bromopropyltyrosine *tert*-butyl ester (**2**): K_2CO_3 (829 mg, 6.00 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** [20] (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 ($CDCl_3$) d 1.41 (s, 9H), 1.42 (s, 9H), 2.28–2.32 (m, *J* = 6.18, 2H), 2.96–3.03 (m, 2H), 3.59–3.61 (t, *J* = 6.42, 2H), 4.07–4.09 (t, *J* = 5.88, 2H), 4.40–4.41 (q, *J* = 7.68, 1H), 4.96–4.97 (d, *J* = 7.98, 1H), 6.82–6.83 (d, *J* = 8.58, 2H), 7.07–7.08 (d, *J* = 8.64, 2H). ^{13}C NMR ($CDCl_3$) d 28.01, 28.35, 29.99, 32.42, 37.60, 54.96, 79.60, 81.93, 114.44, 114.64, 128.74, 130.51, 155.11, 157.68, 171.03. IR (KBr) 3374, 2977, 2923, 1716, 1612, 1504, 1365, 1245, 1160, 1037, 922, 844. HRMS (FAB) m/z Calcd for $C_{21}H_{32}BrNO_5$ $[M + H]^+$ 480.1362. Found: 480.1389.

N-*tert*-Butoxycarbonyl-*O*-{3-[2-(3,4-dimethoxycinnamoylamino)benzoyloxy]

propyl}tyrosine *tert*-butyl ester (**3**): K_2CO_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 add 5% succinic acid (20 mL) and the solution was extracted with CH_2Cl_2 (10 mL × 3), 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 ($CDCl_3$) d 1.409 (s, 3H),

1.412 (s, 3H), 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* = 6.00, 2H), 4.38–4.41 (q, *J* = 7.38, 1H), 4.55–4.57 (t, *J* = 6.30, 2H), 4.95–4.96 (d, *J* = 7.86, 1H), 6.47–6.50 (d, *J* = 15.48, 1H), 6.82–6.84 (dd, *J* = 6.81, 1.86, 1.68, 2H), 6.88–6.89 (d, *J* = 8.28, 1H), 7.07–7.11 (m, 4H), 7.15–7.17 (dd, *J* = 8.28, 1.92, 1H), 7.57–7.60 (dt, *J* = 7.89, 1.62, 1.32, 2H), 7.69–7.71 (d, *J* = 15.54, 1H), 8.05–8.07 (dd, *J* = 7.98, 1.56, 1H), 8.87–8.89 (dd, *J* = 8.52, 0.90, 1H). ^{13}C NMR ($CDCl_3$) d 28.00, 28.34, 28.69, 37.59, 54.97, 55.99, 56.04, 62.39, 64.33, 79.61, 81.95, 109.77, 111.11, 114.38, 114.88, 119.67, 120.69, 122.41, 122.64, 127.11, 128.80,130.60, 130.82, 134.81, 142.12, 142.31, 149.27, 150.96, 155.10, 157.67, 164.77, 168.50, 171.02. IR (KBr) 3301, 2973, 2599, 2321, 1708, 1592, 1515, 1450, 1361, 1253, 1153, 1029, 844, 759, 528. HRMS (FAB) m/z Calcd. for $C_{39}H_{49}N_2O_{10}$ $[M + H]^+$ 705.3382. Found: 705.3392.

O-{3-[2-(3,4-dimethoxycinnamoylamino)benzoyloxy]propyl}tyrosine hydrochloride (TNL-T•HCl): Compound **3** (70.0 mg, 0.0993 mmol) was suspended in 1.0 ml of CH_2Cl_2 and cooled with ice. 50% TFA in CH_2Cl_2 (1.2 mL) was gradually added until the final concentration of TFA reached 27%, and the mixture was stirred at room temperature for 12 h. Then water was added, and the solution was washed with AcOEt (20 mL). A white precipitate deposited was collected and dried under reduced pressure to give a gray solid TNL-T (47% yield). The obtained TNL-T (26.0 mg, 0.474 mmol) was dissolved in MeOH (34 mL), and after confirming that it was completely dissolved, 6N HCl (6.0 mL) was added, and the mixture was stirred for 30 minutes. The mixture was concentrated under reduced pressure to give a precipitate that was collected and dried to give TNL-T•HCl as a pale yellow solid (quantitative yield). mp: 213–215 °C. ^{1}H-NMR (DMSO) δ 2.19 (quin, *J* = 6.2, 2H), 3.03 (m, 2H), 3.81 (s, 3H), 3.83 (s, 3H), 4.09 (t, *J* = 6.1, 1H), 4.11 (t, *J* = 6.1, 2H), 4.46 (t, *J* = 6.2, 2H), 6.82 (d, *J* = 15.5, 1H), 6.89 (d, *J* = 8.7, 2H), 7.01-7.00 (d, *J* = 8.3. 1H), 7.15 (d, *J* = 8.2, 2H), 7.20–7.25 (m, 2H), 7.35 (d, *J* = 1.9, 1H), 7.56 (d, *J* = 15.5. 1H), 7.64 (dt, *J* = 7.9, 1.6, 1H), 7.98 (dd, *J* = 7.9, 1.6, 1H), 8.26 (bs, 2H), 8.40 (dd, *J* = 8.3, 0.7, 1H), 10.8 (s, 1H). ^{13}C NMR(DMSO) δ 28.5, 35.3, 53.8, 56.1, 56.1, 62.7, 64.9, 110.8, 112.1, 115.0, 118.3, 120.1, 121.9, 123.1, 123.7, 127.3, 127.8, 131.1, 131.1, 134.4, 140.4, 142.1, 149.5, 151.2, 158.1, 164.7, 167.6, 170.8. IR (KBr) 3700 – 2500, 2360, 1731, 1677, 1596, 1519, 1459, 1253, 1145, 1091, 1033, 964, 829, 755, 671. HRMS (FAB) m/z Calcd. for $C_{30}H_{33}O_8N_2$ $[M + H]^+$ 549.2231. Found: 549.2245.

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 mL/well; the other: 1.0 × 10^5 cells/500 mL/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 [21].

In vitro**scratch assay**

Cells were seeded in a 24-well plate (1.0 × 10^5 cells/500 mL/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 [22].

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 1 h. TGF-β (10 ng/mL) (R&D Systems Minneapolis) was then added to the 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 mg 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).

Declarations

Data availability

NMR spectra of TNL-T aredepicted in the Supporting Information.

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Conflict of interest The authors declare no competing interests.

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Figures

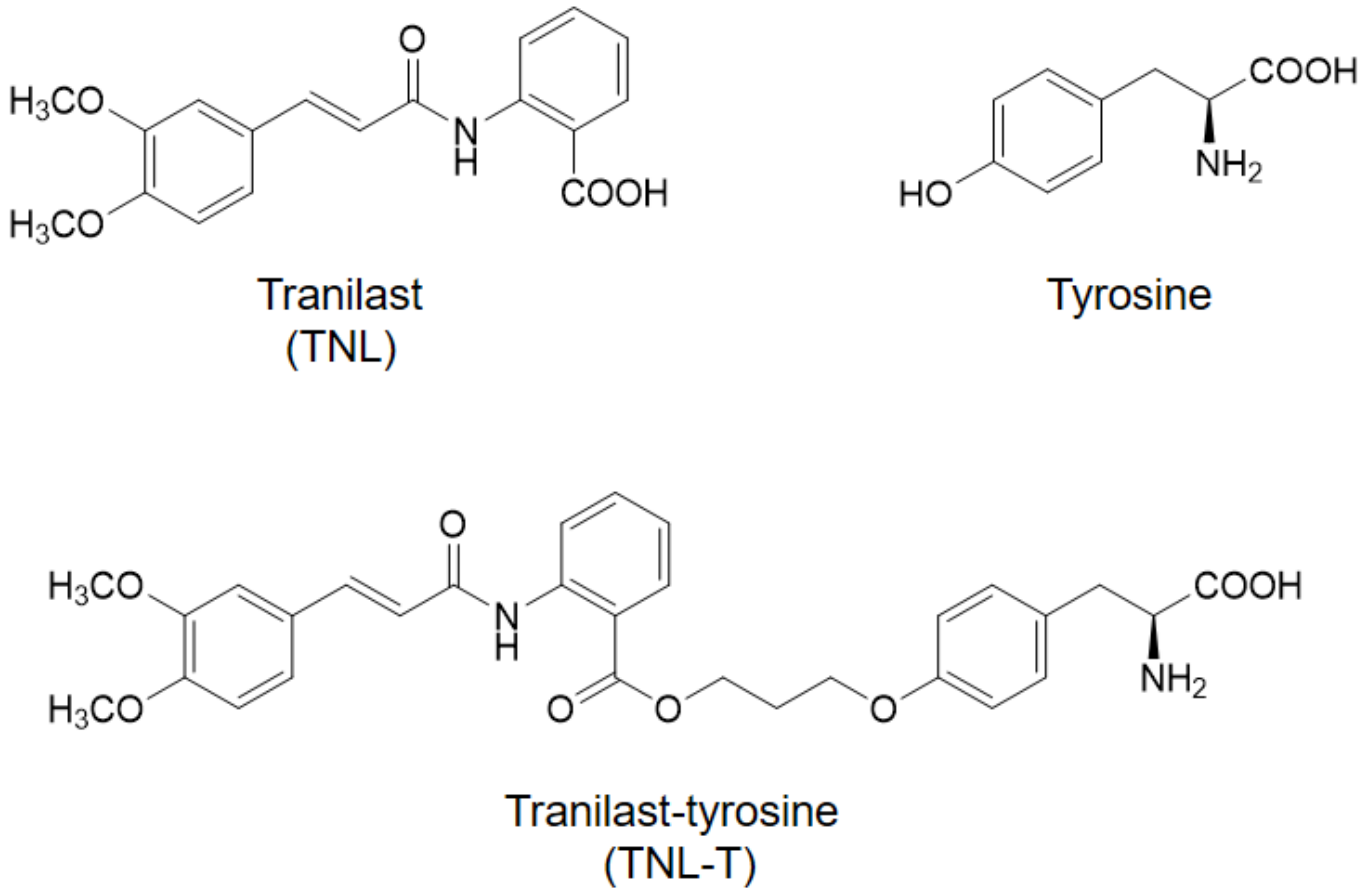

Figure 1

Structures of tranilast (TNL), tyrosine, and the synthesized conjugate (tranilast-tyrosine: TNL-T).

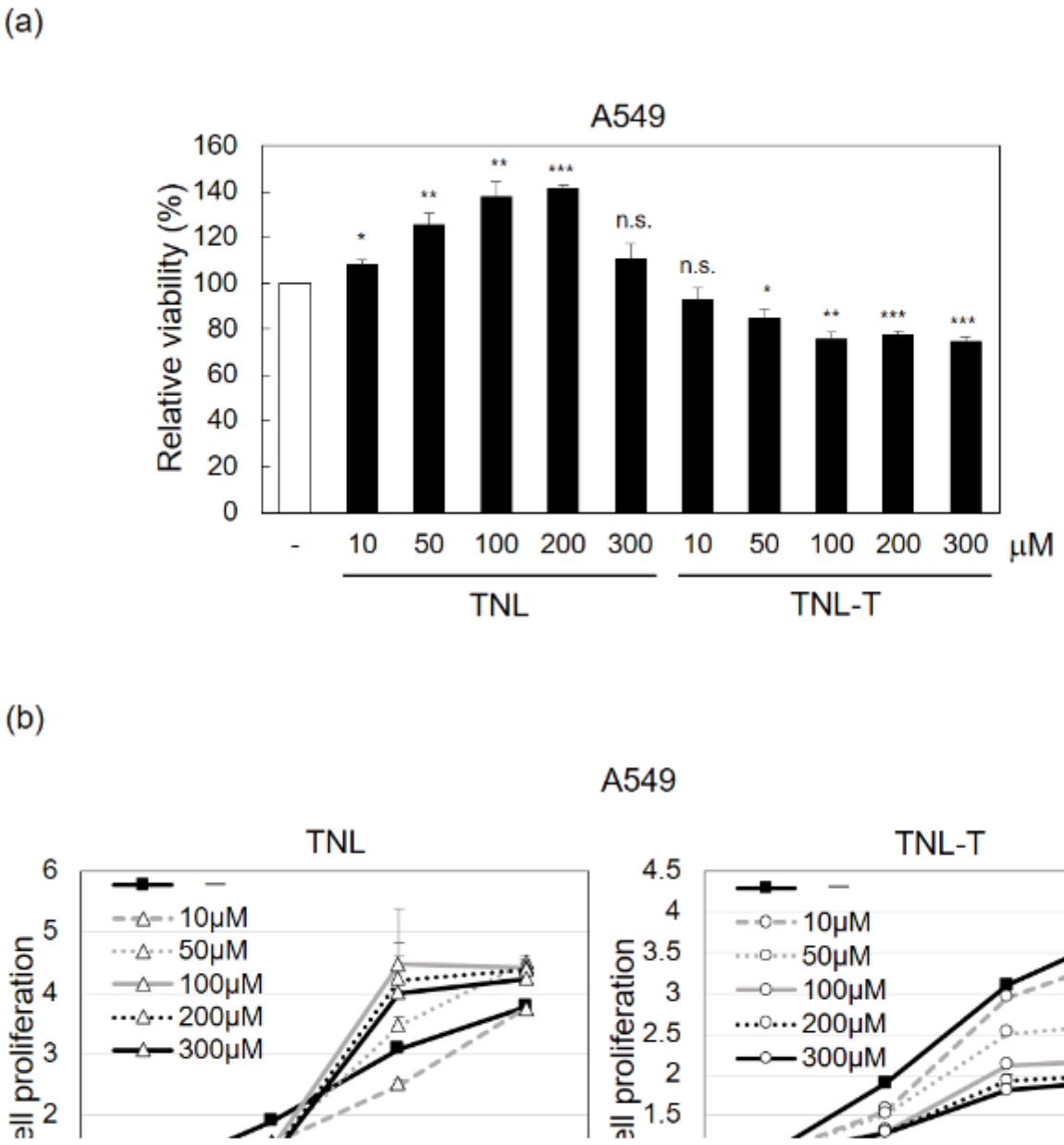

Figure 2

Cytotoxicity of TNL and TNL-T in A549 cells. (a) Cell viability. The cells (1.0×10^5 cells/well) were seeded and incubated for 1 day. Then, the cells were further incubated for 3 days in the presence of test compounds at designated concentrations, and an MTT assay was performed. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the sample without drugs, n.s.: not significant compared with the sample without drugs. (b) Growth curve of cells. The cells (0.2×10^5 cells/well) were seeded and incubated for 1 day. Then, the cells were further incubated for 3 days in the presence of test compounds at designated

concentrations. The MTT assay was performed at the starting point and every other day. Changes in cell viability are shown. TNL, tranilast; TNL-T, tranilast-tyrosine conjugate

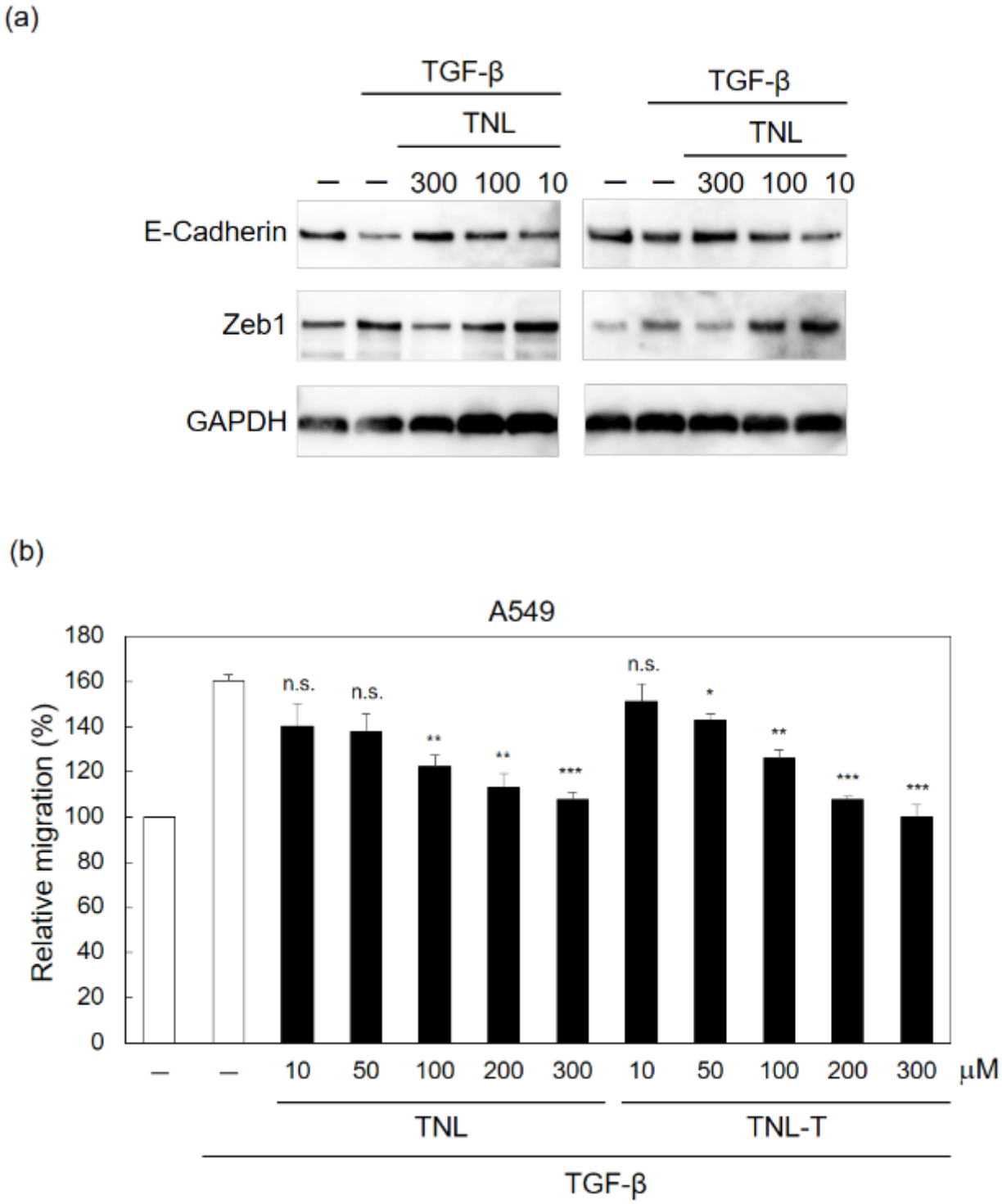

Figure 3

Anti-EMT activities of TNL and TNL-T in A549 cells. (a) Expression of EMT marker proteins. TGF-b-stimulated cells were incubated for 3 days in the presence of test compounds at designated concentrations, followed by cell lysis. The total protein content of lysates was normalized to 10 mg, and

these were analyzed by immunoblotting using E-cadherin and Zeb1 antibodies. (b) Cell migration. *In vitro* scratch assay was performed; that is, the TGF-b-stimulated cells with a scraped part were incubated for 1 day in the presence of test compounds at designated concentrations, and cell migration was evaluated. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the sample without drugs, n.s.: not significant compared with the sample without drugs. EMT, epithelial-mesenchymal transition; TNL, tranilast; TNL-T, tranilast-tyrosine conjugate.

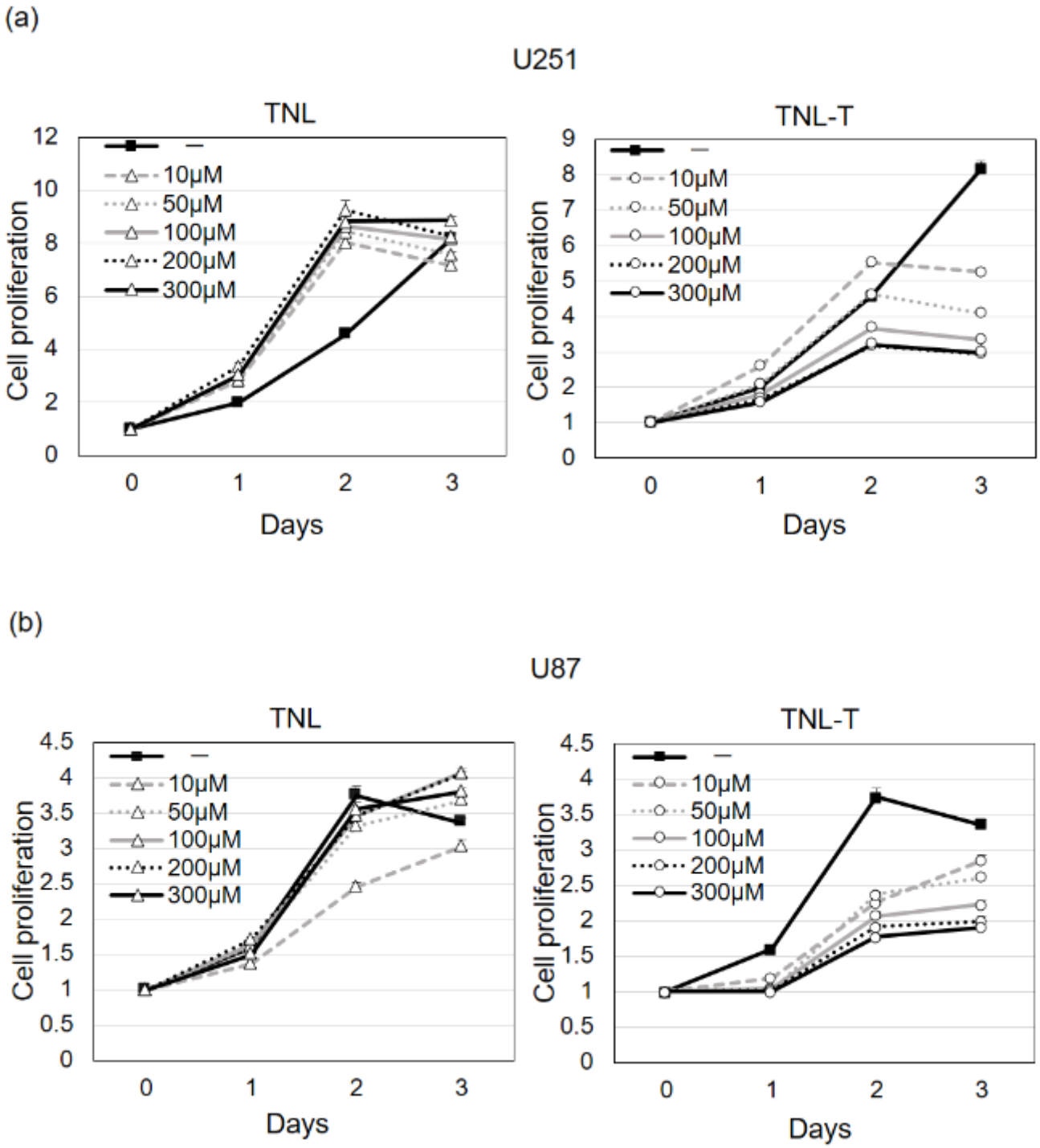

Figure 4

Cytotoxicity of TNL and TNL-T in glioblastoma cells. Growth curves of U251 (a) and U87 (b) cells. This experiment is similar to that shown in Fig. 2b. TNL, tranilast; TNL-T, tranilast-tyrosine conjugate.

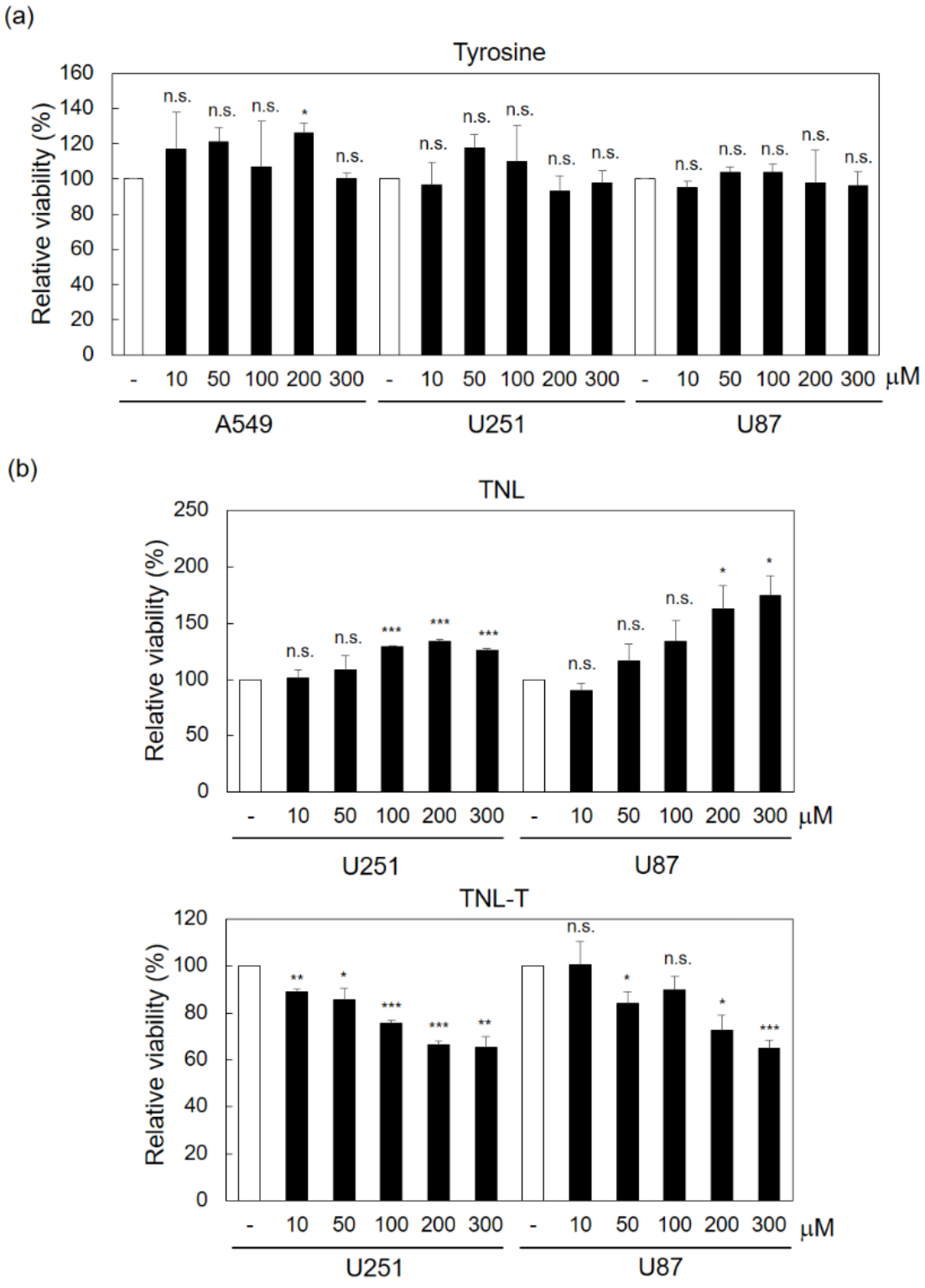

Figure 5

Cytotoxicity of tyrosine. Cell viability of tyrosine (a), TNL (b), and TNL-T (b) in A549/U251/U87 cells. This experiment is similar to that shown in Fig. 2a. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the

sample without drugs, n.s.: not significant compared with the sample without drugs. TNL, tranilast; TNL-T, tranilast-tyrosine conjugate.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Scheme1.png
- Tomaetal.MCRSIfinal.docx