Monoterpene Glycoside ESK246 from *Pittosporum* Targets LAT3 Amino Acid Transport and Prostate Cancer Cell Growth

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Supporting Information

**ABSTRACT:** The l-type amino acid transporter (LAT) family consists of four members (LAT1–4) that mediate uptake of neutral amino acids including leucine. Leucine is not only important as a building block for proteins, but plays a critical role in mTORC1 signaling leading to protein translation. As such, LAT family members are commonly upregulated in cancer in order to fuel increased protein translation and cell growth. To identify potential LAT-specific inhibitors, we established a function-based high-throughput screen using a prefractionated natural product library. We identified and purified two novel monoterpene glycosides, ESK242 and ESK246, sourced from a Queensland collection of the plant *Pittosporum venulosum*. Using *Xenopus laevis* oocytes expressing individual LAT family members, we demonstrated that ESK246 preferentially inhibits leucine transport via LAT3, while ESK242 inhibits both LAT1 and LAT3. We further show in LNCaP prostate cancer cells that ESK246 is a potent (IC$_{50}$ = 8.12 μM) inhibitor of leucine uptake, leading to reduced mTORC1 signaling, cell cycle protein expression and cell proliferation. Our study suggests that ESK246 is a LAT3 inhibitor that can be used to study LAT3 function and upon which new antiprostate cancer therapies may be based.

LATs have been shown to be critical mediators of protein translation and cell growth in a variety of cancers. In prostate cancer, we have shown increased LAT3 expression in primary cancer and increased LAT1 expression in metastasis. Knockdown of either LAT3 or LAT1 expression in prostate cancer cell lines inhibits mTORC1 pathway activation, cell growth, and cell cycle both in vitro and in vivo.

The leucine analogue 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH) is commonly used to inhibit l-type amino acid transport in vitro. However, BCH targets all members of the LAT family and is used in millimolar quantities, making it unsuitable as a LAT inhibitor in vivo. A novel LAT1 inhibitor, JPH203/KYT-0353, has also been reported. This tyrosine analogue has only been tested on LAT1 and LAT2, showing selectivity for LAT1-mediated transport, as well as suppression of HT-29 colorectal adenocarcinoma cell growth in vitro and in vivo.

In this study, we screened a prefractionated natural product library and identified two novel LAT inhibitors, ESK242 and...
We further demonstrate that ESK246 preferentially inhibits LAT3, reducing LAT3-mediated leucine uptake in *Xenopus laevis* oocytes. We show that ESK246 is a more potent LAT inhibitor than ESK242, reducing leucine uptake, mTORC1 signaling, cell cycle protein expression, and proliferation in prostate cancer cell lines.

**RESULTS AND DISCUSSION**

High-throughput Screening of the Prefractionated Nature Bank Library. To discover LAT3-specific inhibitors, we used a function-based strategy for high-throughput screening (HTS) of the Nature Bank prefractionated library (Figure 1). Our high-throughput screen incorporated a 15 min [3H]-L-leucine uptake assay using the androgen-responsive prostate cancer cell line, LNCaP (Figure 1A). The HTS screen was performed on a subset of the Nature Bank lead-like enhanced (LLE) fraction library. This library consists of over 200,000 semipurified fractions sourced from plants and marine invertebrates collected from Australia, China and Papua New Guinea. The HTS involved screening the Nature Bank library, initially analyzing 4488 fractions (51 plates x 88 fractions) for activity against LAT3-mediated [3H]-l-leucine uptake (Figure 1B). Each plate contained 3 negative control wells (DMSO, 0.5% (v/v)) and 3 wells of the LAT family inhibitor BCH (10 mM) as a positive control. BCH consistently inhibited leucine uptake to 30–40% of control, while the negative control ranged from 90–110% on each plate (Figure 1B). In order to reduce the number of hits, we restricted our analysis from the initial screen to fractions that reduced leucine uptake to less than 70% of control, which resulted in a total of 31 fractions (0.7% of analyzed fractions). These fractions were retested in LNCaP cells using the [3H]-l-leucine uptake assay as well as a cell viability assay to determine whether they could inhibit prostate cancer cell viability. Fraction 11711.8-21-11 showed substantial inhibition of both leucine uptake and cell growth (Supporting Information (SI) Figure S1A). As some fractions may contain toxic compounds that rapidly damage cell membranes, such as detergents, leading to low [3H]-l-leucine levels and cell growth, we also examined cell morphology after 48 h treatment, confirming that fraction 11711.8-21-11 did not damage the cell membranes (SI Figure S1B). Fraction 11711.8-21-11 originated from a collection of the plant *Pittosporum venulosum* (Queensland, Australia) and yielded two new LAT inhibitors, ESK242 and ESK246 (Figure 2A). The structures of these new natural products were identified by extensive spectroscopic and spectrometric analyses, confirmed by chemical synthesis, and characterized by a variety of biological methods.

**Isolation and Identification of ESK242 and ESK246.**

ESK246 (trivial name venuloside A) was isolated as an optically active clear oil ([α]D +39, c 0.1, MeOH) with a molecular formula C23H36O7, as established from HRESIMS measurements. The 1H NMR spectrum of ESK 246 in C6D6 was well resolved and showed 21 resonances composed of 2 sp2-hybridized methines, 6 sp3-hybridized methines, 3 diastereotopic methylene pairs, and 7 sp3-hybridized methyls (SI Table S1). The 13C NMR spectrum showed two carbonyls (δC 168.9, 165.8), three other quaternary carbons (δC 157.7, 133.6 and 79.4), two olefinic resonances (δC 121.4 and 116.4), four oxymethines (δC 95.9, 73.8, 70.4, 70.3, and 70.0), one other methine (δC 44.4), three methyls (δC 31.3, 26.96, and 24.0), and seven methyls (δC 27.02, 23.9, 23.53, 23.52, 20.7, 20.2, and 16.6). Interpretation of 2D NMR data allowed for the identification of four partial...
structures depicted in Figure 2B, namely an acetyl substituent (fragment A), a 3-methylbut-2-enoyl group (fragment B), a sugar moiety (fragment C), and a ten-carbon aglycone unit (fragment D). Characteristic chemical shifts for the methyl (δ_H 1.87, 3H, s, δ_C 20.7) and carbonyl resonances (δ_C 165.8, C-1‴) were used to assign fragment A as the acetyl substituent. Fragment B had two vinyl methyl resonances (δ_H 2.14, d, J = 1.0 Hz, δ_C 27.02 and 1.45, d, J = 1.0 Hz; δ_C 20.7), coupled to each other and to an olefin proton (δ_H 5.77, 1H, s, δ_C 116.4), in addition to a quaternary carbon (δ_H 157.7) and an ester carbonyl resonance (δ_C 165.8) consistent with a senecioyl (3-methylbut-2-enoyl) moiety. Partial structure C contained an anomeric proton, four other oxymethines and a doublet methyl resonance, consistent with the presence of a six-deoxy sugar moiety. The 1H NMR chemical shifts and coupling constants for H-1′ (δ_H 4.47, d, J = 7.8 Hz), H-2′ (δ_H 5.66, dd, J = 10.3, 7.8 Hz), H-3′ (δ_H 5.16, dd, J = 10.4, 3.2 Hz), H-4′ (3.72, br d, J = 3.2 Hz), H-5′ (3.10, br q, J = 6.3 Hz), and H-6′-Me (1.17, d, J = 6.3 Hz) protons, together with the ROESY data showed that the glycosyl moiety was β-fucopyranoside. The downfield shift of H-2′ and H-3′ resonances suggested that the C-2 and C-3 hydroxyl groups on the sugar moiety were esterified. Chemical shifts assigned to the acetyl and senecioyl substituents were positioned on C-2′ and C-3′ respectively following crucial 1H−13C HMBC correlations from the H-2′ glycidosic resonance to C-1″ carbonyl on the acetyl group, as well as the glycidosic H-3′ to C-1″ carbonyl on the senecioyl group (Figure 2B). The sugar residue in ESK246 was therefore concluded to be 2′-acetyl-3′-senecioyl-β-fucopyranoside. Signals for the aglycone fragment D, C_10H_17 as suggested by the molecular formula, were composed of an olefinic multiplet, three diastereotopic methylene pairs, a single methane and three methyl resonances, consistent with the presence of an α-terpineol moiety. Crucial HMBC correlation from the anomeric H-1’ to the quaternary C-8 carbon (δ_C 79.4) (Figure 2B) connected substructure D to the sugar unit to complete the planar structure of the glycoside.

The absolute configuration of the natural product was determined via comparison of chiro-optical and NMR spectral data with that of the synthetically prepared analogues. Hydrolysis of ESK246 in 5% HCl in MeOH afforded a mixture of methyl-3-O-senecioyl-β-fucopyranoside, methyl-3-O-senecioyl-α-fucopyranoside and methyl-4-O-senecioyl-α-fucopyranoside (see Supporting Information). Comparison of the optical rotation values of the ESK246 hydrolysis-sourced α- and β-anomers of methyl-3-O-senecioyl-fucopyranoside with that of the synthetic α- and β-anomers of methyl-3-O-senecioyl-fucopyranoside established the absolute configuration of fucose residue as δ (see Supporting Information). The δ-configuration of the fucose sugar is typical of plant-sourced glycosides, whereas l has been reported from bacteria- and plant-sourced polysaccharides, as well as glycolipids and glycoproteins of animal origin.26 Resolving the absolute configuration of the α-terpineol proved to be a challenge as the acid hydrolysis work-up and isolation did not yield any terpineol product. The absolute configuration of the natural product was therefore resolved with total synthesis of the racemic and (4S)-α-terpineol diastereomers of ESK246. Starting from d-fucose and the readily available (4Rαc)- and (4S)-α-terpineols, a four-step synthesis was achieved (Figure 2C and Supporting Information text). Koenigs−Knorr glycosylation of bromo-fucosyl donor with α-terpineol afforded the fully protected α-terpineol fucoside, which was subsequently deprotected under basic conditions (NaOMe/MeOH) to give the α-terpineol fucoside. Utilizing the inherent difference in reactivity of the fucose hydroxyl groups,27 selective acylation at the 3′O position of α-terpineol fucoside with 3,3-dimethylacryloyl chloride was achieved.28 Introduction of the acetate moiety at the 2′O position was carried out under basic conditions using acetic anhydride to give the final product. In the 1H NMR spectrum of the C-4 epimeric mixture of ESK246, the greatest signal dispersion between the two isomers was observed with the H-9 and H-10 resonances (SI Figure S2). These two sets of resonances were therefore used as a point of
determination in the determination of the absolute configuration of ESK246. In an 1H NMR-based titration experiment depicted in SI Figure S3, addition of the naturally occurring ESK246 to the C-4 epimeric mixture of α-terpineol-8-O-β-D-(2′-acetyl, 3′-senecioyl) fucopyranoside resulted in the enhancement of the resonances associated with the (4R)-α-terpineol epimer, therefore securing the stereochemistry of the natural product as (4R)-α-terpineol-8-O-β-D-(2′-acetyl, 3′-senecioyl) fucopyranoside. ESK242 (trivial name venuloside B) was isolated as an optically active clear oil ([α]D +20, c 0.1, MeOH) with a molecular formula C23H30O6, as established from HRESIMS measurements. The compound was isomeric with ESK246 and showed an identical number of 1H and 13C resonances in the NMR spectra. The most significant differences between the NMR spectra of the two constitutional isomers were centered on the two methyl vinyl resonances H-4‴ (δH 2.05, m; δC 16.0) and H-5‴ (δH 1.94, m; δC 20.8) as well as an olefinic methine resonance at H-3‴ (δH 5.79, m; δC 139.3). In contrast to ESK246, which had a seneiol glycosyl group at C-3, ESK242 showed two methyl vinyl resonances each coupled to an olefin proton, consistent with a 1,2-dimethyl vinyl group. A ROESY experiment (τmix = 400 ms) assigned the geometry of the double bond to be cis, based on the observation of a correlation between H-3‴ and H-5‴ methyl resonances, consistent with the presence of an angeloyl group. Crucial 1H-13C HMBC correlations from the glycosidic H-3‴ to C-1‴ placed the angeloyl group on C-3‴ of the sugar moiety. Interpretation of 1D and 2D NMR data (SI Table S1) established the structure of ESK242 as α-terpineol-2′-acetate-3′-angeloyl-β-D-fucopyranoside. Based on the structural homology with ESK246 and the close correspondence of the NMR chemical shifts (SI Table S1) the absolute configuration of ESK242 is proposed to be (4R)-α-terpineol-8-O-β-D-(2′-acetyl, 3′-angeloyl) fucopyranoside.

Effects of ESK242 and ESK246 on Leucine Uptake in Prostate Cancer Cell Lines. To examine the inhibitory effects of ESK242 and ESK246, we next investigated the impact of these new compounds on LNCaP and PC-3 prostate cancer cell lines. We have previously shown that LAT3 is the dominant leucine transporter in LNCaP cells, while LAT1 plays a more important role in PC-3 cells (SI Figure S4A).16 In the presence of compounds ESK242 and ESK246, leucine uptake was decreased in a dose-dependent manner in LNCaP cells (Figure 3A). The IC50 of ESK242 is 29.6 ± 1.2 μM, while the IC50 of ESK246 is 8.12 ± 1.2 μM. Both compounds had an IC50 more than 2 orders of magnitude lower than the universal LAT inhibitor, BCH, whose IC50 is 4060 ± 1.1 μM (Figure 3A). In contrast, neither compound substantially inhibited leucine uptake at the same concentration in PC-3 cells, in which LAT1 is more abundantly expressed (SI Figure S4B).

Characterization of ESK242 and ESK246 by Amino Acid Uptake Assay in Oocytes. The differential effects on leucine uptake in LNCaP and PC-3 cells suggested these compounds selectively inhibit individual LAT family members. To test the specificity of the purified compounds, we expressed LAT1/4F2hc, LAT2/4F2hc, LAT3 or LAT4 in Xenopus laevis oocytes and [3H]-leucine uptake assays were performed. After 4 days, transporters were expressed on the plasma membrane of oocytes, as shown by an increase in [3H]-leucine uptake compared to uninjected oocytes (SI Figure S5A). To demonstrate expression of the LAT3 transporter on the surface of oocytes, we performed a surface biotinylation assay, which confirmed LAT3 expression in injected oocytes only (SI Figure S5B). The optimal concentration of leucine, and duration of leucine uptake, was determined by dose response and time course experiments, showing 1 mM L-leucine for 30 min to be optimal (SI Figures S5C and D). After treatment with 50 μM ESK242 or ESK246 for 30 min, LAT1/4F2hc transport activity was significantly inhibited by ESK246 (34.2%; Figure 3B). LAT3-mediated leucine transport was significantly inhibited by both ESK242 (47.9%) and ESK246 (47.3%; Figure 3D). Neither compound significantly inhibited leucine uptake mediated by LAT2/4F2hc (Figure 3C) or LAT4 (Figure 3E). These results suggest that ESK246 is a preferential LAT3 inhibitor and ESK242 inhibits both LAT1 and LAT3. We next determined the IC50 of both compounds for LAT3 in oocytes. The IC50 of ESK246 was calculated at 146.7 ± 2.4 μM, which is lower than the IC50 of ESK242 at 281.8 ± 1.3 μM (Figure 4A), suggesting that ESK246 has a higher affinity for LAT3 than ESK242.

In order to investigate the nature of inhibition of LAT3 by ESK242 and ESK246, leucine dose responses were performed in the absence or presence of 50 μM and 500 μM of each inhibitor. As the concentration of inhibitor increases, the Km of leucine for LAT3 (6.3 ± 0.5 mM) increases slightly in the presence of 50 μM ESK242 (7.5 ± 0.5 mM) but decreases to 2.7 ± 0.5 mM in the presence of 500 μM ESK242 (Figure 4B). In the presence of 50 μM and 500 μM ESK246, the Km of leucine for LAT3 decreases to 4.7 ± 0.6 mM and 2.1 ± 0.3 mM, respectively (Figure 4C) suggesting a competitive mechanism of action for these compounds. The maximal rate of uptake also decreases as the inhibitor concentration increases, which would not be expected for a purely competitive inhibitor suggesting that these compounds may be acting in a mixed competitive/noncompetitive manner. An Eadie–Hofstee transformation of the data (Figure 4B and C, inset)
also demonstrates that both ESK242 and ESK246 are acting as mixed inhibitors.

**Effects of ESK242 and ESK246 on mTORC1 Signaling and Cell Growth in Prostate Cancer Cell Lines.** Having established that ESK242 and ESK246 inhibit leucine uptake in LNCaP cells with an IC$_{50}$ between 8–30 μM (Figure 3A), we next assessed the downstream effects of leucine deprivation on signaling and cell growth. Leucine uptake mediated by LAT3 has been shown to regulate mTORC1 activation in prostate cancer cells.16 Both ESK242 and ESK246 suppressed phosphorylation (activation) of the mTORC1 target protein p70S6K (Figure 5A), consistent with a mechanism consequent to inhibition of LAT3-mediated leucine transport in LNCaP cells. We next examined cell viability in the presence of compounds using an MTT assay in prostate cancer cells. Both ESK242 and ESK246 led to lower cell viability in LNCaP cells after 3 days treatment (Figure 5B). Only compound ESK242, but not ESK246, significantly inhibited cell viability in PC-3 cells, (SI Figure S6A), which may be due to ESK242 inhibition of both LAT1 and LAT3. To determine whether decreased cell viability is due to activation of apoptosis or suppression of proliferation, we first examined apoptosis using flow cytometry to detect “flipped” Annexin-V protein levels in the plasma membrane. Cells exposed to UV showed increased Annexin-V flipping indicative of apoptosis (Figure 5C). Neither ESK242 nor ESK246 induced apoptosis, consistent with previous studies examining BCH (Figure 5C).16 Therefore, it is likely that the effects on viability are mediated through inhibition of cell cycle and proliferation. To test this, we next examined BrdU (bromodeoxyuridine, a thymidine analogue) incorporation in DNA using flow cytometry and showed the BrdU incorporation rate was decreased after BCH, ESK242, or ESK246 treatment (Figure 5D,E). Compound ESK246 showed the highest level of inhibition of BrdU incorporation (4.9%; 17.2% of control), which was significantly lower than either BCH (14.7%; 51.6% of control).

**Figure 4.** ESK242 and ESK246 inhibit LAT3 via a mixed mode of inhibition. (A) Increasing doses of ESK242 and ESK246 inhibit [3H]-l-leucine transport in oocytes expressing LAT3 with IC$_{50}$ values of 281.8 ± 1.3 μM and 146.7 ± 2.4 μM, respectively. (B–C) [3H]-l-leucine dose response in the absence or presence of 50 μM or 500 μM ESK242 (B) or ESK246 (C). Data are fitted to the Michaelis–Menten equation and normalized to the maximal rate of transport of [3H]-l-leucine alone. An Eadie–Hofstee transformation was performed (inset, B–C). Data show mean ± SEM (n = 5).

**Figure 5.** Effects of ESK242 and ESK246 in LNCaP cells. (A) Representative Western blots (from n = 3) of p70S6K phosphorylation after BCH (10 mM), ESK242 (50 μM), and ESK246 (50 μM) inhibition. GAPDH was used as the loading control. (B) MTT cell viability assay (n = 3) in LNCaP cells incubated with BCH (10 mM), ESK242 (50 μM) and ESK246 (50 μM). Two-way ANOVA test was performed, **P < 0.01, ***P < 0.001. (C) Analysis of apoptosis (n = 3) in LNCaP cells using Annexin-V and PI staining after inhibition with BCH (10 mM), ESK242 (50 μM), and ESK246 (50 μM). (D,E) Analysis of cell proliferation using BrdU incorporation in LNCaP cells inhibited with BCH (10 mM), ESK242 (50 μM) and ESK246 (50 μM). Representative flow cytometry analysis (D) and quantification (E) from 3 separate experiments are shown. One-way ANOVA test was performed, *P < 0.05, **P < 0.01, ***P < 0.001.
or ESK242 treatment (23.5%; 82.7% of control; Figure 5E). Our previous studies have shown that reduced extracellular amino acid levels inhibit expression of the cell cycle regulator CDK1 and the ubiquitination enzyme UBE2C in prostate cancer and melanoma. Therefore, we examined expression of CDK1 and UBE2C in the presence of BCH, ESK242, and ESK246, showing expression of both proteins was down regulated by ESK246 (SI Figure S6B).

**Conclusions.** We have utilized a function-based drug discovery platform that enabled high-throughput screening of LAT3-specific natural products from the Nature Bank fraction library. Using this platform, we identified two novel monoterpenoid glycosides, ESK242, a dual LAT1 and LAT3 inhibitor, and ESK246, which is more selective for inhibition of LAT3 in both oocytes and LNCAp cells. Notably, ESK246, inhibits LAT-mediated leucine transport in LNCAp cells with a ~500-fold lower IC50 compared to the leucine analogue BCH. We anticipate that derivatives of these inhibitors could be used as prostate cancer therapeutics as well as to investigate the biological function of LAT1 and LAT3 during development and in disease.

**METHODS**

**General Chemical Experimental Procedures.** UV spectra were recorded on a Jasco V650 UV/vis spectrophotometer. NMR spectra were recorded at 30 °C on either a Varian 500 or 600 MHz Unity INOVA spectrometer. The latter spectrometer was equipped with a triple resonance cold probe. The 1H and 13C NMR chemical shifts were referenced to the solvent peaks for benzene-d6 at δH 7.20 and δC 128.0, respectively, and for DMSO-d6 at δH 2.50 and δC 39.43, respectively. HRESIMS were recorded on a Bruker DaLtonics Apex III 4.7e Fourier-transform mass spectrometer. Silica gel chromatography was performed using Merck silica gel 60 (0.015–0.040 mm). A Waters 600 pump equipped with a Waters 996 PDA detector and a Waters 717 autosampler were used for HPLC. A Phenomenon Oxynx monolythic column [100 × 10 mm] was used for semipreparative HPLC separations. All solvents used for chromatography, UV, and MS were Lab-Sанb HPLC grade (RCI Lab-Scan, Bangkok, Thailand), and the H2O was Millipore Milli-Q FF filtered. All starting materials and reagents for the synthesis were obtained from commercial suppliers, Sigma-Aldrich, Carbosynth, Alfa Aesar, Merck Millipore, and used without further purification. High-performance liquid chromatography (HPLC) grade solvents were obtained from Labscan, and purified using a PureSolv MD 5 solvent purification system from Innovative Technology. Reactions were performed in flame-dried glassware under positive N2 pressure, with magnetic stirring. Rubber septa and syringes were used for liquid transfers. Thin layer chromatography (TLC) was performed on 0.25 mm Merck silica gel 60 F254 plates and plates visualized by staining with Seebach’s stain; phosphomolybdic acid/cerium(IV) sulfate 2.5:1 in H2O/H2SO4 94:6 (mL).

**Plant Material.** *Pittosporum venulosum* (F. Muell) (Nature Bank code 11711.8) was collected in July 1995 from State Forest 144, Mt. Pittosporum venulosum containing 1:1 LAT1/4F2hc, LAT2/4F2hc, LAT3, or LAT4 cRNA, which is more selective for inhibition of LAT3 in both oocytes and LNCAp cells. DMSO 0.5% (v/v) was used as the negative control and 10 mM BCh was used as the positive control. Cells were collected, transferred to filter paper using a 96-well plate harvester (Wallac PerkinElmer), dried, exposed to scintillation fluid, and counts measured using a liquid scintillation counter (PerkinElmer).

**Cell Viability Assay.** Cells in exponential growth phase were harvested and seeded (1 × 105/well) in a flat-bottomed 96-well plate. The cells were incubated overnight in RPMI media, prior to culture with or without each inhibitor. MTX solution (10 μM, 3-[(4S,5S)-3,4-dihydroxyphenyl] tetraethylammonium bromide; Millipore) was added to each well for 4 h, prior to addition of 100 μL of isopropanol/HCl solution and mixed thoroughly. The plates were immediately read at 570 nm/630 nm in a PolarStar plate reader (BMG). Results were plotted as percentages of the absorbance observed in control wells (vehicle/DMSO).

**Oocyte Uptake Assay.** Human LAT1, LAT2, LAT3, LAT4, and 4F2hc cDNAs were subcloned into the oocyte transcription vector pOV, respectively. The resulting transporter cDNAs were linearized with SpeI. Complementary RNA (cRNA) was transcribed with T7 RNA polymerase and capped with 5′-7-methylguanosine using the mMessage mMACHINE kit (Ambion). All cRNAs were purified using a NucAway Spin Column (Ambion).

Stage V oocytes were harvested from *Xenopus laevis* as described previously, and all surgical procedures followed a protocol approved under the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Oocytes were injected with 23 nL of cRNA mix containing 1:1 LAT1/4F2hc, LAT2/4F2hc, LAT3, or LAT4 cRNA, respectively. The cells were incubated overnight in RPMI media, prior to culture with or without each inhibitor. MTX solution (10 μM, 3-[(4S,5S)-3,4-dihydroxyphenyl] tetraethylammonium bromide; Millipore) was added to each well for 4 h, prior to addition of 100 μL of isopropanol/HCl solution and mixed thoroughly. The plates were immediately read at 570 nm/630 nm in a PolarStar plate reader (BMG). Results were plotted as percentages of the absorbance observed in control wells (vehicle/DMSO).

**Biotinylation Assay.** Surface proteins on *Xenopus laevis* oocytes were isolated using the Cell Surface Protein Isolation Kit (Thermo Scientific Pierce). The oocytes were incubated for 30 min at 4 °C on an
orbital shaker with Sulfo-NHS-SS-biotin. After three washes with PBS, the oocytes were sonicated in 500 μL of Lysis buffer containing protease inhibitors. The lysate was incubated on ice for 30 min and spun for 2 min at 10 000g. The labeled proteins were isolated from the supernatant using NeutrAvidin agarose and eluted with SDS-PAGE sample buffer containing 50 mM DTT. The eluted proteins were then analyzed by Western blotting as detailed below.

**Brdu Incorporation Assay.** Cells were seeded at a density of 2 × 10^5 in 6-well plates and allowed to adhere overnight. After serum starvation, cells were incubated with either DMSO, 10 mM BCH, 50 μM ESK242, or 50 μM ESK246 for 22 h. At the end of the treatment, BrdU (150 μg/mL) was added to culture media and incubated for another 2 h, followed by detachment using Tryple (Life Technologies). Cells were fixed and stained using the Becton Dickinson APC BrdU flow cytometry kit (BD). The BrdU antibody was diluted 1 in 50. Nuclei were counterstained with 7-AAD (7-aminoactinomycin). The cells were analyzed on a Canto II flow cytometer (BD) with postanalysis performed using FlowJo software (Tree Star Inc.).

**Apoptosis Assay.** Cells were seeded at a density of 2 × 10^5 in 6-well plates, allowed to adhere overnight, before incubation with DMSO, 10 mM BCH, 50 μM ESK242, or 50 μM ESK246 for 48 h, respectively. Positive control group cells were irradiated in a UV Stratalinker 2400 (Stratagene) with a 400 μjy dosage and incubated in fresh media for 16 h. Cells were detached using Tryple and resuspended in 1 mL of binding buffer (HEPES-buffered PBS supplemented with 2.5 mM calcium chloride) containing antiannexin V-APC (BD) and incubated for 15 min in the dark at RT. PI solution (20 μg/mL) was added, and the cells were analyzed on a Canto II flow cytometer (BD) with postanalysis performed using FlowJo software.

**Western Blots.** Cells were seeded at a density of 2 × 10^5 in 6-well plates, allowed to adhere overnight, before incubation with DMSO, 10 mM BCH, 50 μM ESK242, or 50 μM ESK246 for 6 h or 3 d. Cells were lysed by the addition of lysis buffer (200 μL) with protease inhibitor Cocktail III (Bioprocessing Biochemical) and 1 mM Na3VO4 (Sigma). Equal protein (micro-BCA method; Pierce, IL) was loaded on 4–12% gradient gels (Life Technologies), electrophoresed, and transferred to PVDF membrane. The membrane was blocked with 2.5% (w/v) BSA in PBS-Tween20, and incubated with the primary and secondary antibodies. The secondary HRP-labeled antibodies were detected using enhanced chemiluminescence reagents (Pierce) on 4%–13% gradient gels (Life Technologies), electrophoresed, and transferred to PVDF membrane containing protease inhibitor Cocktail III. The labeled proteins were isolated from the supernatant and identified by mass spectrometric analysis. The identities of the natural compounds were confirmed by comparison with authentic standards or by comparison with spectra recorded in our laboratory. The structure of the natural products was elucidated by 1D and 2D NMR spectroscopy and MS analysis.

**Statistical Analysis.** Data are expressed as mean ± SEM. Experiments were performed in triplicate, except where noted in the Figure Legend. All data were analyzed using a one-way ANOVA test, apart from MTT assays that used a two-way ANOVA test in GraphPad Prism v6 (GraphPad Software, Inc.).

**ASSOCIATED CONTENT**

* Supporting Information
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* AUTHOR INFORMATION

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**Author Contributions**
R.J.Q. and J.H. designed the experiments. Q.W. and J.H. screened the Nature Bank library in LNCap cells. T.G. isolated and identified the structure of the natural products. S.B. and R.H.P. synthesized the natural products. C.G.B. and J.F. cloned the cDNA of transporters. J.F. performed the oocyte assays. Q.W. and A.M. examined the compounds in LNCap cells. Q.W., T.G., S.B., R.H.P., J.F., R.M.R., J.E.J.R., M.J., R.J.Q., and J.H. interpreted results and edited the manuscript. Q.W., T.G., S.B., R.H.P., J.F., C.G.B, R.J.Q., and J.H. wrote the paper.

**Notes**
The authors declare no competing financial interest.

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