Supporting Information

Largazole is a Brain-Penetrant Class I HDAC Inhibitor with Extended Applicability to Glioblastoma and CNS Diseases

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Contents **Page**

Methods

Synthesis of Largazole

The synthesis was carried out similarly as previously described.[1,2] Briefly, subunits were synthesized and assembled to form a linear precursor which upon macrocyclization reaction results in a 16-membered cyclic depsipeptide core. This cyclic core was then subjected to olefin cross-metathesis to install the thioester moiety.

Biological Materials and Methods

Cell culture. SF268 and SF295 astrocytoma-glioma cells were cultured in Roswell Park Memorial Institute medium (RMPI 1640, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, MilliporeSigma) and 1% antibiotic-antimycotic (Invitrogen). SH-SY5Y neuroblastoma cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Thermo Fisher Scientific) supplemented with 15% FBS (MilliporeSigma) and 1% antibiotic-antimycotic (Invitrogen). Cells were incubated at 37 °C humidified air and 5% CO_2.

Cell viability assay. SF268 and SF295 cells were plated at 5000 cells/well while SH-SY5Y were plated at 40,000 cells/well in 96-well plates. After 24 h incubation, cells were treated with various concentrations of largazole or solvent control. After 48 h, cell viability was measured using MTT according to the manufacturer's instructions (Promega).

***In vivo* functional studies.** The experiment was carried out as described by Salvador et al.[3] Briefly, the mice obtained from Charles River Laboratories Inc. (Wilmington, MA) and used for human tumor xenografts were 3 to 5 weeks old female nude mice (*nu/nu*). Mice were treated with largazole or solvent control by intraperitoneal injection (in DMSO, 25 µL/mouse) or oral gavage (in 60% polyethylene glycol, 15% glycerol, 15% ethanol, 10% DMSO, 100 µL/mouse). Brains were harvested at 4, 12, and 24 h following the treatment. All studies were carried out under the protocol approved by the Institutional Animal Care and Use Committee at the University of Florida (study #201502564).

Acute toxicity studies. The nontumored animal toxicity study was performed with athymic nude mice through the Developmental Therapeutics Program at the NCI. Single ip doses of largazole (200 mg/mL stock in DMSO) of 100 mg/kg (inj vol 0.5 µL/g body wt), 200 mg/kg (inj vol 1 µL/g body wt), and 400 mg/kg ((inj vol 2 µL/g body wt) were given and mice monitored for 15 days, suggesting a maximum tolerated dose (MTD) of 200 mg/kg. Toxicity was only observed at 400 mg/kg, although the DMSO concentrations was also higher than usually employed in efficacy studies (25 µL/mouse ~ 1 µL/g body wt DMSO). Repeated ip dosing, ranging from 60 to 75 mg/kg, in hollow fiber screening assays (QD x 4) with 12 different tumor cell lines also did not show signs of toxicity.

Immunoblot analysis. Brain samples were homogenized through sonication in PhosphoSafe lysis buffer, centrifuged, and the supernatants were collected and used for immunoblot analysis probing with acetyl histone H3 antibody (Lys9/14) obtained from Millipore. Protein concentrations were measured using the BCA protein Assay kit (Pierce). Samples containing equal amounts of protein were separated by SDS-PAGE gel (4–12%), transferred to polyvinylidene difluoride (PVDF) membranes, probed with antibodies and detected with the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

RNA extraction, reverse transcription, and real time quantitative polymerase chain reaction (qPCR). RNA was extracted from largazole treated mouse brain tissues using TRIzol reagent according

to the manufacturer's standard protocol (Ambion by life technologies). For cellular studies, the cells (SF268: 100,000 cells/well; SF295: 100,000 cells/well) were seeded in 6-well plates and following 24 h incubation, RNA was isolated using RNeasy Mini Kit (Qiagen). SuperScript II Reverse Transcriptase and Oligo (dT) (Invitrogen) were used for cDNA synthesis. Following reverse transcription, qPCR was performed on a reaction solution (25 µL) prepared using 1 µL aliquot of cDNA, 12.5 µL of TaqMan 2× universal master mix, 1.25 µL of 20X TaqMan gene expression assay probe, and 10.25 µL of RNase-free water. The qPCR experiment was carried out using ABI 7300 sequence detection system with the following thermocycler program: 2 min at 50 °C, 10 min at 95 °C, and 15 s at 95 °C (40 cycles), and 1 min at 60 °C. The target genes used were *BDNF* (Hs00380947_m1) and *Bdnf* (Mm04230607_s1), while the endogenous control probes used were *GAPDH* (Hs02758991_g1) and Actin (Mm02619580_g1).

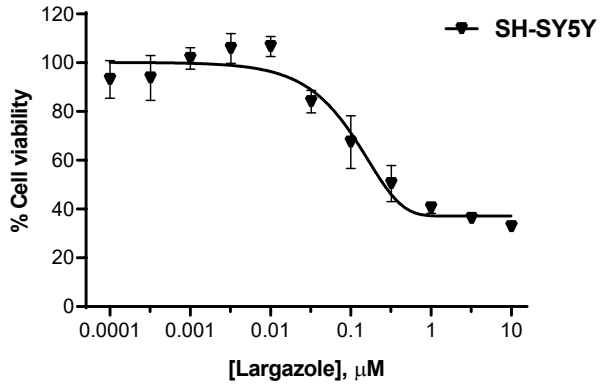
RNA-sequencing. The Interdisciplinary Center for Biotechnology Research (ICBR) Gene Expression Core, University of Florida (UF) carried out the Illumina RNA library construction and subsequently the NextSeq500 sequencing (Illumina) as described in details by Bousquet et al.[4] NanoDrop Spectrophotometer (NanoDrop Technologies, Inc.) was used for Quantitation and Agilent 2100 Bioanalyzer (Agilent Technologies, Inc) was used to assess sample quality. ClonTech RiboGone – Mammalian-Low input ribosomal RNA removal kit for Human, Mouse and Rat Samples (cat#: 634848) was used to remove rRNAs following the manufacturer's protocol. The depleted RNA were then used for library construction using SMARTer Universal Low input RNA kit for sequencing (cat#: 634940) combined with Illumina Nextera DNA Library Preparation Kit (cat#: FC-121-1030) according to the manufacturer's instructions. Libraries were quantitated by Bioanalyzer and qPCR (Kapa Biosystems, catalog number: KK4824). Finally, the libraries were pooled equal molar concentration and sequenced by Illumina 2X75 NextSeq 500 as described by Bousquet et al.[4] Data are deposited in SRA database, accession number PRJNA533817.

Metabolite Analyses

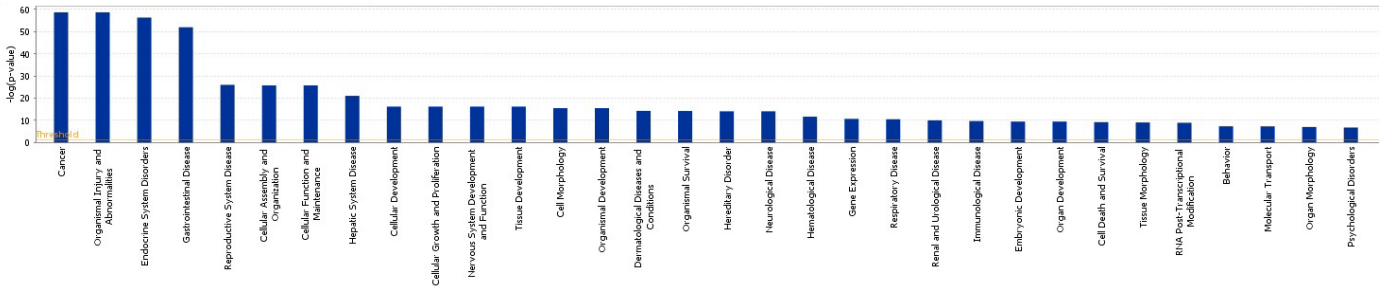
General. Analysis of largazole thiol in brains was performed using an HPLC-MS [3200 QTRAP (Applied Biosystems) equipped with a Shimadzu UFLC System] as described.[5]

HPLC-MS parameters. Column, Onyx Monolithic C18 (3.0 × 100 mm), Phenomenex; solvent, 0.1% aqueous formic acid (solvent A) – 0.1% formic acid in MeOH (solvent B); flow rate, 0.5 mL/min; detection by ESIMS in positive ion mode (MRM scan); gradient:40% A and 60% B, then increased to 83% B at 4 min and decreased to 60% B at 5 min. The retention times (t_R, min; MRM ion pair) of the analytes and internal standard were as follows: harmine (1.5; 213→170) and largazole thiol (2.1; 497.2→141). The compound dependent parameters were as follows: Largazole thiol: DP 49.9, EP 5.3, CE 45.4, CXP 4.13, CEP 17, Harmine: DP 45, EP 5.3, CE 45.4, CXP 4.13, CEP 17.0. Source gas parameters were as follows: CUR 10, CAD Low, IS 4500, TEM 450.0, GS1 50.0, GS2 40.0.

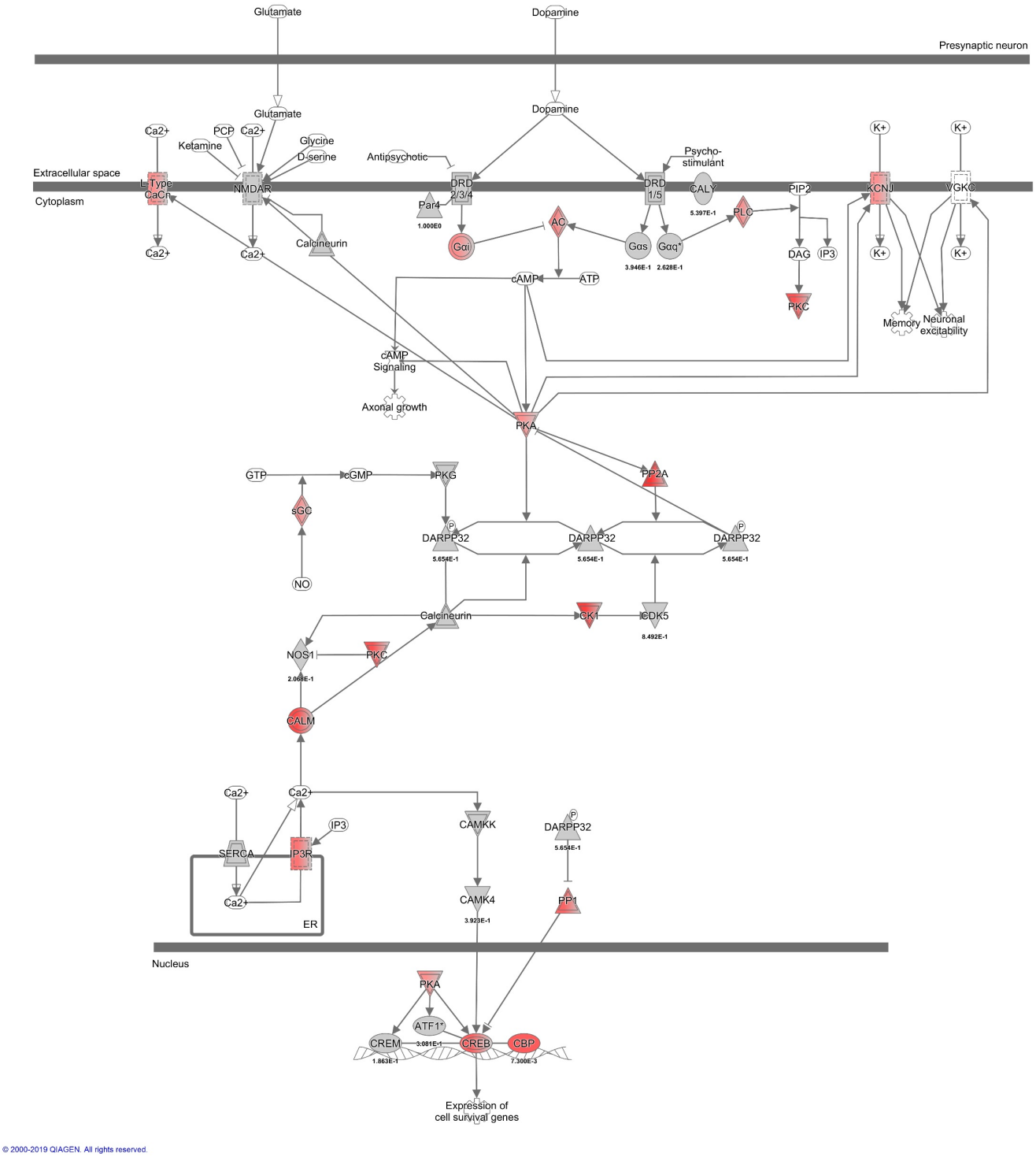
***In vivo* largazole thiol levels in brains.** The brains were harvested, homogenized and extracted with methanol (1:10 w:v) spiked with internal standard harmine and subsequently incubated for 10 min (on ice) followed by centrifugation at 16,000*g* (15 min at 4 °C). The pellet was then re-extracted from the supernatant with methanol. The supernatants were pooled, dried under nitrogen and partitioned between ethyl acetate and water. The organic layer was then collected, dried and reconstituted in 50 µL MeOH. A 10 µL aliquot was injected for HPLC-MS analysis according to the validated method of Yu et al.[5] Briefly, the analysis was carried out using MRM where largazole thiol along with the internal standard harmine were specifically monitored in the samples using the aforementioned compound and mass dependent parameters.



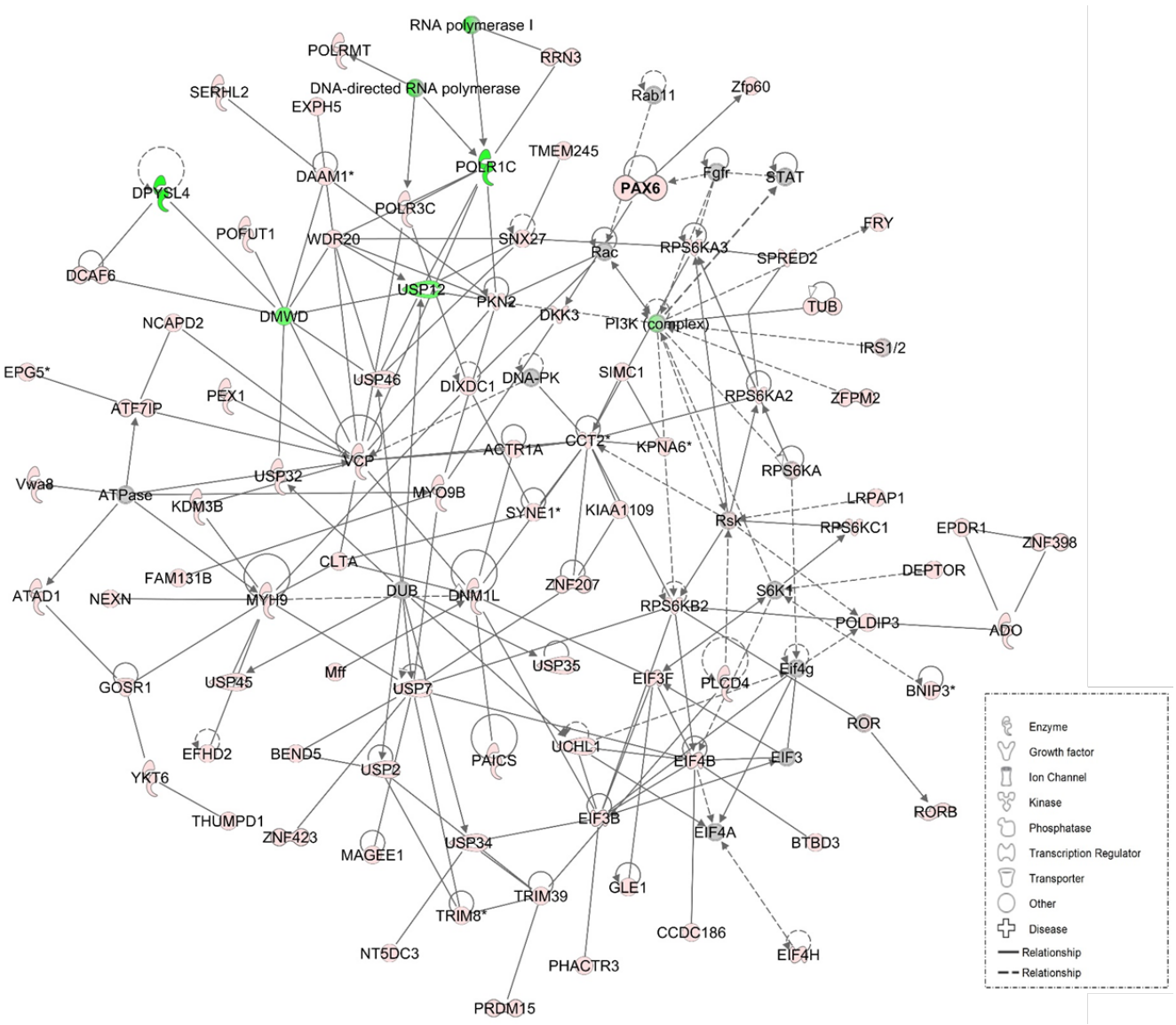
Supplementary Figure S1. The antiproliferative effects of largazole against SH-SY5Y neuroblastoma cells assessed by MTT assay (48 h). Error bars represent SD, n = 3.



Supplementary Figure S2. Top diseases and functions identified through the analysis of RNA-sequencing data using IPA (2.5-fold cutoff, $P < 0.05$).



Supplementary Figure S3. Dopamine-DARPP32 feedback in cAMP signaling as a top canonical pathway identified by RNA-sequencing experiment. Analysis was carried out using IPA (2.5-fold cutoff, $P < 0.05$).



Supplementary Figure S4. Overlay of top molecular networks affected by largazole, identified through the analysis of RNA-sequencing data of mice brains (50 mg/kg ip, 12 h treatment) using IPA (2.5-fold cutoff, $P < 0.05$). The networks are implicated in cancer, cell cycle, gene expression, protein synthesis, posttranslational modifications, and cellular assembly and organization. Red fill = upregulated, green fill = downregulated. PAX6 that is also a major node in the neuro networks (Figure 4) and emphasized in bold font.

Table S1. Cancer related applications predicted to be decreased in response to treatment (based on IPA; 2.5-fold cutoff, $P < 0.05$).

Diseases or functions annotation	p-Value	Activation z-score	No. of molecules	Selected genes
Head and neck cancer	3.76E-52	-2.0	907	AKT3, MAPK9, SOD2
Extraadrenal retroperitoneal tumor	5.87E-17	-2.538	367	HDAC4, IGF1R, CXCL12
Urinary tract tumor	9.64E-11	-3.0	231	CDK12, CXCL12, HDAC4

Supplementary References

(1) Ying, Y.; Taori, K.; Kim, H.; Hong, J.; Luesch, H. Total Synthesis and Molecular Target of Largazole, a Histone Deacetylase Inhibitor. *J. Am. Chem. Soc.* **2008**, *130* (26), 8455–8459.

(2) Chen, Q.-Y.; Chaturvedi, P. R.; Luesch, H. Process Development and Scale-up Total Synthesis of Largazole, a Potent Class I Histone Deacetylase Inhibitor. *Org. Process Res. Dev.* **2018**, *22* (2), 190–199.

(3) Salvador, L. A.; Park, H.; Al-Awadhi, F. H.; Liu, Y.; Kim, B.; Zeller, S. L.; Chen, Q. Y.; Hong, J.; Luesch, H. Modulation of Activity Profiles for Largazole-Based HDAC Inhibitors through Alteration of Prodrug Properties. *ACS Med. Chem. Lett.* **2014**, *5* (8), 905–910.

(4) Bousquet, M. S.; Ratnayake, R.; Pope, J. L.; Chen, Q.-Y.; Zhu, F.; Chen, S.; Carney, T. J.; Gharaibeh, R. Z.; Jobin, C.; Paul, V. J.; Luesch, H. Seaweed Natural Products Modify the Host Inflammatory Response via Nrf2 Signaling and Alter Colon Microbiota Composition and Gene Expression. *Free Radical Biol. Med.* **2020**, *146*, 306-323.

(5) Yu, M.; Salvador, L. A.; Sy, S. K. B.; Tang, Y.; Singh, R. S. P.; Chen, Q.-Y.; Liu, Y.; Hong, J.; Derendorf, H.; Luesch, H. Largazole Pharmacokinetics in Rats by LC-MS/MS. *Mar. Drugs* **2014**, *12* (3), 1623–1640.