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Supporting Information

Psoromic Acid Derivatives: A New Family of Small-Molecule Pre-mRNA Splicing Inhibitors Discovered by a Stage-Specific High-Throughput in Vitro Splicing Assay

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Supporting Information

Methods

Cell culture and the generation of stable cell lines

HeLa S3 cells (Computer Cell Culture Centre, Belgium) were grown essentially as described previously.[1] To generate FLAG/HA-tagged Abstrakt, cDNA encoding full-length Abstrakt protein was cloned into a modified pIRESneo plasmid (Clontech) containing an N-terminal FLAG/HA-tag[2] via PCR with the primer pair 5'-Abstrakt (5'-GCTAGCGGCCGCATGGAGGAGTCGGAACCCGA-3') and 3'-Abstrakt (5'-TAGCGAATTCATCAGAAGTCCATGGAGCTGTG-3'). For cloning, the restriction sites NotI and EcoRI were used to generate the expression vector pIRESneo-FLAG/HA-Abstrakt. The generation of stable cell lines expressing FLAG/HA-tagged Abstrakt or hSmu-1 was performed essentially as described by Malik and Roeder.[2] HeLa S3 cells were seeded into a 6-well plate to a density of 5×10^5 cells per well in DMEM/NCS (2.5 mL/10%) and incubated overnight at 37°C (5% CO_2, 95% humidity). Subsequently, HeLa S3 cells were transfected with pIRESneo-FLAG/HA-Abstrakt using the transfection reagent FuGENE HD (Roche) according to the manufacturer's instructions. After incubation overnight, cells from one 6-well were transferred to three 10 cm cell culture dishes. To select for stable transformants, cells were subsequently cultivated in DMEM/10% NCS containing G418 (1.5 mg/mL, Geneticin, Gibco BRL). The medium was exchanged after each 48 h. After 10 days, colonies of cells that incorporated the expression vector (enabling Neomycin resistance) were formed. Individual colonies were transferred to a 24 well-plate with each well containing selection medium (1 mL). Cells were cultivated to semi-confluency, transferred to 6-well plates. Approximately 10^5 cells were incubated in SDS-loading buffer for 10 min at 95°C and analysed by western blotting with anti-FLAG antibodies (Sigma) for the presence of FLAG/HA-tagged Abstrakt protein. Clones that stably expressed FLAG/HA-tagged Abstrakt were used for further cultivation and for the production of nuclear extracts. The cell line stably expressing FLAG-tagged hSmu-1 protein was generated in essentially the same way. Nuclear extracts were prepared according to Dignam et al[3].

In vitro splicing reaction in a 384 well format and Z' value determination

PM5 pre-mRNA carrying MS2 aptamers upstream of the 5' exon was prepared by run off transcription and preincubated with an equimolar amount of purified MS2-MBP fusion protein.[4] High binding, black 384well Fluotrac600 microplates (Greiner) were coated with rabbit polyclonal

antibodies to MBP (Abcam) by passive adsorption (0.4 µg/well). PM5 pre-mRNA bound by MS2-MBP was added to the microplate (2 pmol/well) and incubated for at least 1 h. A 20 µL splicing reaction containing HeLa nuclear extract from cells stably expressing FLAG-tagged Abstrakt or hSmu-1 proteins (30%, v/v), monoclonal ANTI-FLAG® M2-Peroxidase (0.15 µg/mL, Sigma), KCl (65 mM), $MgCl_2$ (3 mM), ATP (2 mM), creatine phosphate (20 mM), Tween20 (0.1 %, v/v), and in some cases inhibitors supplied in DMSO (with 2.5 % final concentration of DMSO) was added to each well. After 90 min of incubation (if not otherwise stated) at 30°C, the wells were washed 6 times with 100 µL of HEPES-KOH pH 7.9 (20 mM), $MgCl_2$ (1.5 mM), NaCl (150 mM) and Tween20 (0.1 %). Luminescence was measured using a VICTOR^3 V multilabel reader (PerkinElmer) immediately after addition of 20 µL SuperSignal® ELISA Femto Maximum Sensitivity Substrate (Pierce) per well. Spliceostatin A was kindly provided by M. Yoshida. Garcinol, BA3 and splitomicin were purchased from Alexis Biochemicals and DHC was from Sigma. For Z' value determination we performed the splicing reaction with or without immobilized pre-mRNA within the same microplate and used the following formula:[7]

$$Z' = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} - \mu_{c-}|}$$

Mean of positive control μ_{c+} was 78596 (in relative luminescent units), mean of negative control μ_{c-} 4813, Standard Deviation of positive control σ_{c+} 4152,71, Standard Deviation of negative control σ_{c-} 230,8538, Z'-value 0.82.

High throughput screening of a 30,000 compound library

5 mM stocks of the compound library were pre-diluted to 125 µM with HEPES-KOH pH 7.9 (20 mM) and Tween20 (0.01%) in 384well HiBase clear PS microplates (Greiner) and transferred to Fluotrac600 microplates (Greiner) using a Freedom EVO® liquid handling Robot (programmed with the Freedom EVOware Plus® software from Tecan). The microplate splicing assay was performed as described above except it contained 40% of the pre-dilutions (corresponding to final compound concentrations of 50 µM and 1% DMSO) and was incubated at 26°C. For the handling of antibodies to MBP, nuclear extract and peroxidase substrate solution, a Multidrop combi dispenser (Thermo Scientific) was used. All microplate wash steps were performed with a Power Washer 384 (Tecan). Luminescence readout was measured using a Infinite® F500 multimode reader (Tecan). Screening data analysis was performed with the software AccordHTS and Pipeline Pilot from Accelrys. For primary screening and secondary follow up experiments,

Psoromic and Norstictic acid were obtained from MicroSource. For subsequent experiments Psoromic acid was purchased from ABCR GmbH.'

In vitro splicing, MS2 affinity selection and IC_{50} determination

In vitro transcription of uniformly ^{32}P-labeled MINX pre-mRNA substrate, and subsequent standard splicing reactions were performed as described previously.[5] Splicing complexes were analyzed on a low melting point agarose gel (1.5%, w/v)[4] and visualized by phosphor imaging (Typhoon 8600, GE Healthcare). Spliceosomal complexes were purified using MS2-MBP affinity selection.[4] RNA recovered from splicing reactions and the eluted complexes was analyzed on a 7 M urea/14% (w/v) polyacrylamide gel, and visualized by SYBR® Gold staining (Invitrogen) and phosphor imaging (Typhoon 8600, GE Healthcare). For IC_{50} determination, concentration dependent inhibition measurements were performed in triplicate. Each dilution series of the compounds contained 8 data points within the range from 1 up to 250 µM. For the 100 % activity control, instead of the test compound, 2.5 % of DMSO was added. Amounts of RNA were measured with a phosphor imager (Typhoon 8600, GE Healthcare). Splicing efficiency was calculated by the ratio [mRNA]/([mRNA]+[pre-mRNA]) and expressed as a percentage of the corresponding ratio for the control reaction with DMSO. IC_{50} values were calculated using the Sigmoidal Dose-Response Model in XLfit.

In vitro splicing assay with MS2 affinity-purified spliceosomal complexes

HeLa nuclear extract was treated with micrococcal nuclease (MN) as described previously.[6] In vitro splicing (60 µL of reaction volume) was performed for the indicated times with 10 µL of MS2 affinity-selected splicing complexes (~50 fmol), and 40% buffer D or 40% MN-treated nuclear extract. The RNA was isolated, separated on a 7M urea/14% (w/v) polyacrylamide gel, and visualized by phosphor imaging (Typhoon 8600, GE Healthcare).

References

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Figure S1 Affinity purified complexes can be chased into active spliceosomes: A) Wildtype B complex (lanes 1-10) and complexes accumulated in the presence of 100 µM psoromic acid after 2 h of splicing (lanes 11-20) were incubated for the indicated times under splicing conditions without (lanes 1-5 and 11-15) or with (lanes 6-10 and 16-20) micrococcal nuclease treated nuclear extract (MN-NX). RNA was analysed on a denaturing gel and visualized with a Phosphor Imager. The positions of lariat-intron/3'-exon, lariat-intron, pre-mRNA, fully spliced mRNA, and 5'-exon (top to bottom) are indicated on the right. B) Quantification of the splicing reaction. The intensities of the spliced mRNA and pre-mRNA bands of each lane were quantitated via Phosphor Imaging and plotted as the ratio of spliced mRNA to pre-mRNA (lanes 1-5, black closed circles; lanes 6-10, black open circles; lanes 11-15, green closed circles; lanes 16-20, green open circles).

Figure S2. Psoromic acid derivatives which do not inhibit pre-mRNA splicing.

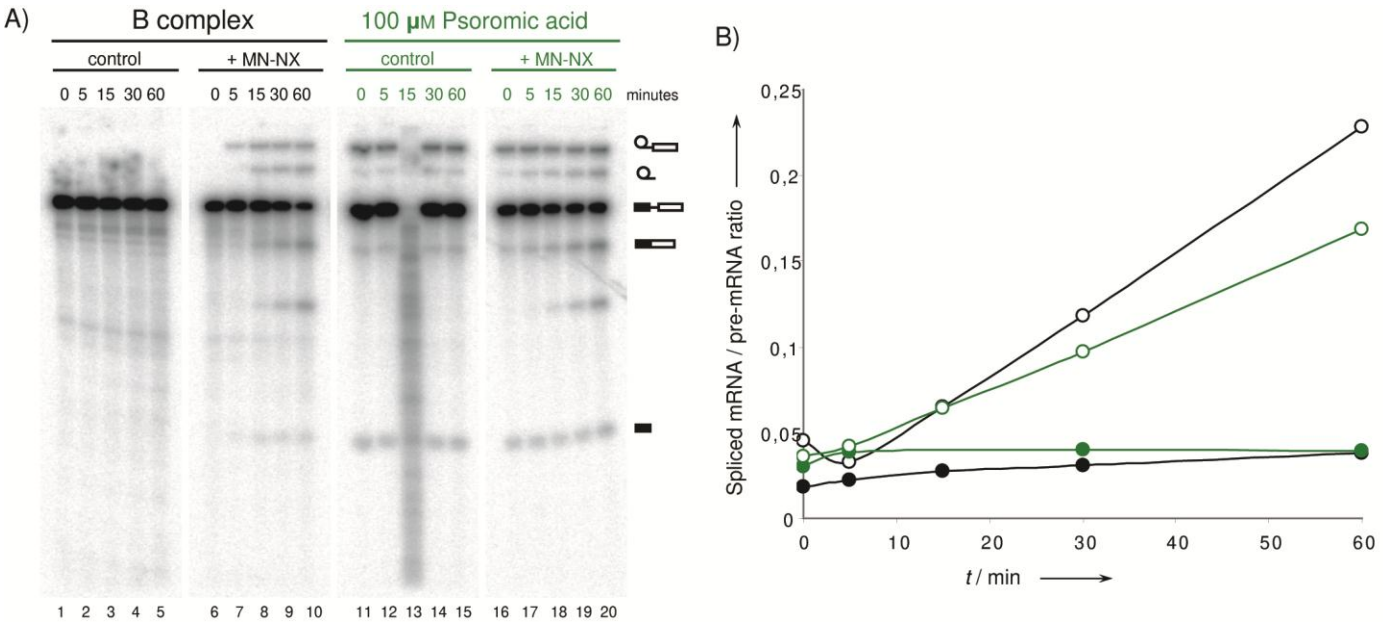

Figure S1.

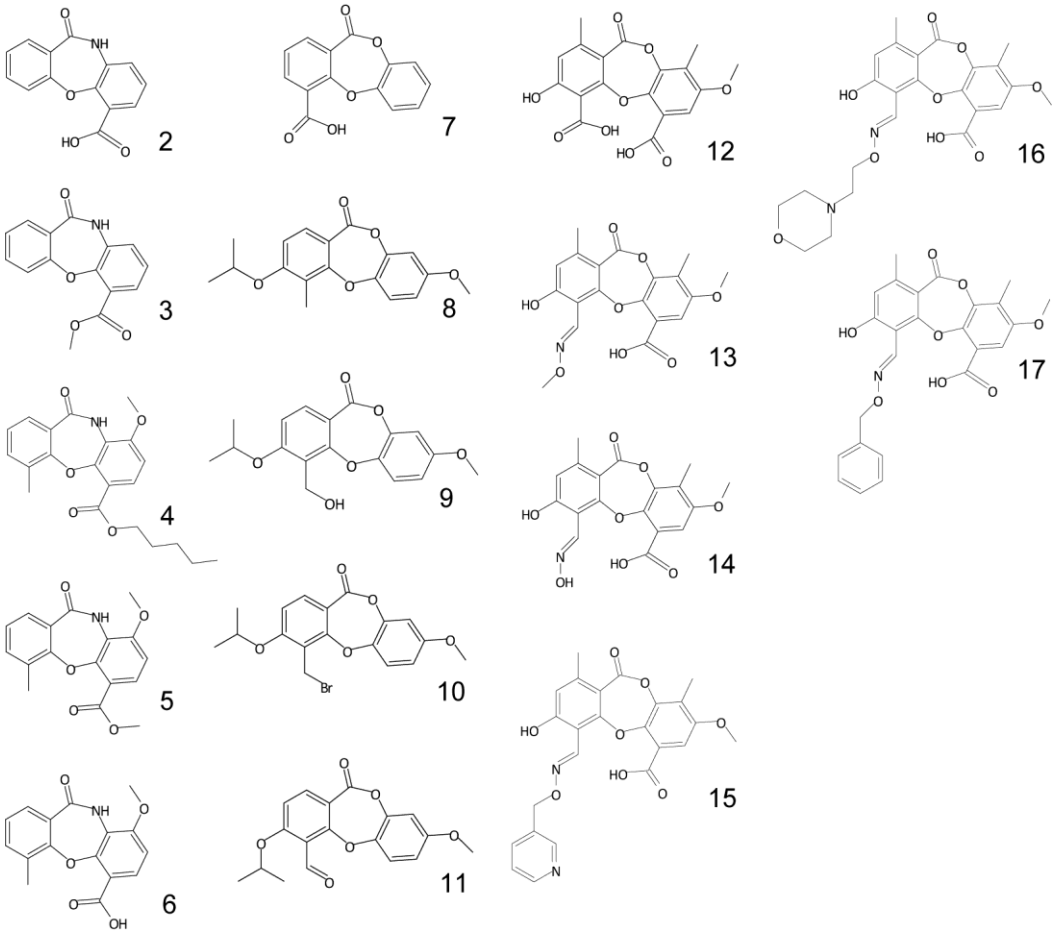

Figure S2.