Supplementary Material

Modulation of Host-Parasite Interactions with Small Molecules
Targeting *Schistosoma mansoni* microRNAs

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Figure S1: RNA secondary structures of the selected pre-miRNAs and the designed small RNA hairpins containing the potential small molecule binding pockets.
Figure S2: Imino proton NMR spectra of the small RNA hairpins in Figure S1. The characteristic resonances of the UUCG tetraloop ($U_L$ and $G_L$) are given and are consistent with the predicted secondary structure.

Table S1: Evaluation of the imino proton spectra and $^{19}$F screening of the small RNA hairpins.

| pre-miRNA | binding pocket | NMR of RNA      | 19F screening (nr of hits) |
|-----------|----------------|-----------------|----------------------------|
| miR-124   | RNA-1          | broad signals, badly dispersed | 3                          |
| miR-124   | RNA-2          | broad signals   | 4                          |
| miR-10    | RNA-3          | broad signals   | 4                          |
| miR-10    | RNA-4          | narrow signals  | 5                          |
| miR-125a  | RNA-5          | broad signals   | 0                          |
| miR-125a  | RNA-6          | narrow signals  | 2                          |
| miR-125a  | RNA-7          | narrow signals  | 2                          |
Table S2: Results of fragment screening of RNA-4 using the poised fragment library. Hits were identified by chemical shift perturbation (CSP). In several cases, fragment binding is accompanied by line broadening. The T2-effect is reported as a reduction in signal height in the 1D NMR spectrum.

| Compounds | CSP (Hz) | T2 | Smiles |
|-----------|---------|----|--------|
| F1        | 6.1     |    | CC(=O)NC=1C=CN=CC1C |
| F2        | 4.1     | 50%| CN|C=1C=CC(=CC1C)=O|NC=2C=CN=CC2 |
| F3        | 3.5     |    | O=C(NCCC=1C=CN=CC1JC=2CCC=CC2) |
| F4        | 2.6     | 20%| CN|IC|CC|C=2CC(CC|CN|)=CC2 |
| F5        | 2.6     |    | CC1|N|N(C)(C)=|C1CC(=O)N |
| F6        | 2.5     | 15%| CS(=O)(=O)N|CC|CC2=CC|CN|=CC2 |
| F7        | 2.0     | 20%| CN|IC|C(=O)=O|C=N1|C|=2C=CN=CC2 |

Synthesis and characterization of trans-1, cis-1, trans-2 and trans-3

Synthesis of intermediate 2-methyl-1H-benzo[d]imidazole-6-carbohydrazide. Step-1: To a stirred solution of 3,4-diaminobenzoic acid (1.2 g, 7.89 mmol) in hydrochloric acid (12 mL, 395.28 mmol), was added acetic acid (3 mL) at 25 °C, and reaction mixture was stirred at reflux temperature for 16 h. After the completion of the reaction, the reaction mixture was evaporated under reduced pressure and neutralized to pH 3-4 using ammonia solution. Solid precipitates were filtered, washed with water and dried to afford 2-methyl-1H-benzo[d]imidazole-6-carboxylic acid (1.26 g, 90.7 %) as a brown solid. LCMS: Calculated for C_{9}H_{8}N_{2}O_{2}, Molecular Weight: 176.18, Observed 177.1 (M+H), RT. 0.861 min, 99.10 % (Max). 1H NMR (300 MHz, DMSO-d_{6}): δ 12.61 (s, 1 H), 8.05 (s, 1 H), 7.77 (dd, J = 8.40 Hz, 1 H), 7.51 (d, J = 8.40 Hz, 1 H), 2.54 (s, 3 H). Step-2: To a stirred solution of 2-methyl-1H-benzo[d]imidazole-6-carboxylic acid (1.2 g, 6.81 mmol) in ethanol (20.0 mL), H_{2}SO_{4} (1.815 mL, 34.1 mmol) was added at 0 °C,
and the reaction mixture was stirred at 90 °C for 16 hours. After the completion of reaction, the reaction mixture was evaporated under reduced pressure, added to water (10.0 mL) and neutralized with 2.0 M sodium bicarbonate solution. Then extracted with dichloromethane (DCM) (30.0 mL), washed with water (10.0 mL) and brine solution (10.0 mL). The organic layer was dried with sodium sulfate, filtered, and the filtrate evaporated under reduced pressure to afford ethyl 2-methyl-1\(H\)-benzo[d]imidazole-6-carboxylate (1.1 g, 79 % yield) as an off-white solid. LCMS: Calculated for C\(_{11}\)H\(_{12}\)N\(_2\)O\(_2\), Molecular Weight: 204.23, Observed 205.1 (M+H), RT. 1.293 min, 99.74 % (Max), \(^{1}\)H NMR (400 MHz, DMSO-d\(_6\)): \(\delta\) 12.54 (d, \(J = 8.40\) Hz, 1 H), 8.11-18.01(m, 1 H), 7.80-7.74 (m, 1 H), 7.58 -7.48 (m, 1 H), 4.32 (q, \(J = 7.20\) Hz, 2 H), 2.54 (s, 3 H), 1.34 (t, \(J = 7.20\) Hz, 3 H). Step-3: To a stirred solution of ethyl 2-methyl-1\(H\)-benzo[d]imidazole-6-carboxylate (1.1 g, 5.39 mmol) in methanol (10.0 mL), were added hydrazine monohydrate (1.584 mL, 32.3 mmol) at 25 °C, and the reaction mixture was stirred at 80 °C for 48 h. After the completion of reaction, the reaction mixture was evaporated under reduced pressure, washed with ethanol (20.0 mL) and dried under reduced pressure to afford 2-methyl-1\(H\)-benzo[d]imidazole-6-carbohydrazide (1.0 g, 98 %) as an off-white solid. LCMS: Calculated for C\(_9\)H\(_{10}\)N\(_4\)O, Molecular Weight: 190.21, Observed 191.2 (M+H), RT. 0.798 min, 79.97 % (Max), \(^{1}\)H NMR (400 MHz, DMSO-d\(_6\)): \(\delta\) 12.43 (s, 1 H), 9.68 (s, 1 H), 7.97 (s, 1 H), 7.65 (d, \(J = 8.40\) Hz, 1 H), 7.46 (s,1H), 4.44 (s, 2 H), 2.54 (s, 3 H). Synthesis of trans-1 & cis-1. Step-4: To a stirred solution of 2-methyl-1\(H\)-benzo[d]imidazole-6-carbohydrazide (300 mg, 1.577 mmol) in ethanol (5.0 mL), were added picolinaldehyde (0.154 mL, 1.577 mmol) and acetic acid (18.94 mg, 0.315 mmol) at 0 °C, and the reaction mixture was stirred at 80 °C for 16 hours. After the completion of reaction, reaction mixture was evaporated under reduced pressure to remove ethanol, washed with methyl tertiary butyl ether (MTBE) (20.0 mL) and dried under reduced pressure to obtain a crude mixture. The mixture was further purified by flash column chromatography using 230-400 mesh silica gel and 15-20% methanol in DCM as eluent to afford trans-1 (50 mg, 10.89 % yield) and cis-1 (90 mg, 20.41 %) as off-white solids. trans-1 LCMS: Calculated for C\(_{15}\)H\(_{13}\)N\(_5\)O, Molecular Weight: 279.30, Observed 280.1 (M+H), RT. 1.406 min, 99.89 % (Max), HPLC: 2.855 min, 95.99% (Max). \(^{1}\)H NMR (400 MHz, DMSO-d\(_6\)): \(\delta\) 12.53 (s, 1 H), 12.01 (s, 1 H), 8.63 (d, \(J = 4.80\) Hz, 1 H), 8.51 (s, 1 H), 8.11 (s, 1 H), 7.99 (d, \(J=5.20\) Hz, 1 H), 7.90 (t, \(J = 8.40\) Hz, 1 H), 7.76 (d, \(J = 8.00\) Hz, 1 H), 7.57 (d, \(J = 8.40\) Hz, 1 H), 7.44-7.40 (m, 1 H), 2.54 (s, 3 H). \(^{13}\)C NMR (176 MHz, DMSO-d\(_6\)) \(\delta\) 164.3, 154.3, 154.0, 150.0, 147.8, 137.3, 126.5, 124.7, 121.8, 120.2, 115.1, 15.2. cis-1 LCMS: Calculated for C\(_{15}\)H\(_{13}\)N\(_5\)O, Molecular Weight: 279.30, Observed 280.1 (M+H), RT. 1.646 min, 99.04 % (Max), HPLC: 3.629 min, 99.206% (Max). \(^{1}\)H NMR (400 MHz, DMSO-d\(_6\)): \(\delta\) 15.56 (s, 1 H), 12.59 (s, 1 H), 8.89 (s, 1 H), 8.14 (t, \(J = 7.60\) Hz, 1 H), 8.06 (s, 1 H), 7.85 (d, \(J = 8.00\) Hz, 1 H), 7.76-7.71 (m, 2 H), 7.63-7.60 (m, 2 H), 2.55 (s, 3 H). \(^{13}\)C NMR (176 MHz, DMSO-d\(_6\)) \(\delta\) 164.0, 154.5, 152.5, 148.7, 139.1, 138.7, 126.9, 126.3, 125.1, 121.0, 114.6, 15.2.
Synthesis of \textit{trans-2}: To a stirred solution of 2-methyl-1\textit{H}-benzo[\textit{d}]imidazole-6-carbohydrazide (300 mg, 1.577 mmol) in ethanol (5.0 mL), were added nicotinaldehyde (0.154 mL, 1.577 mmol) and acetic acid (18.94 mg, 0.315 mmol) at 0 °C, and the reaction mixture was stirred at 80 °C for 16 hours. After completion of reaction, reaction mixture was evaporated under reduced pressure to obtain crude mixture. Then washed with MTBE (20.0 mL), followed by washings with ethanol (10.0 mL), and dried under reduced pressure to afford \textit{trans-2} (300 mg, 66.0 %) as an off-white solid. LCMS: Calculated for C\textsubscript{15}H\textsubscript{13}N\textsubscript{5}O, Molecular Weight: 279.30, Observed 280.1 (M+H), RT. 1.386 min, 99.47 % (Max), HPLC: 3.076 min, 96.88% (Max).\textsuperscript{1}H NMR (400 MHz, DMSO-\textsubscript{d\textsubscript{6}}): δ 12.51 (d, \textit{J} = 18.80 Hz, 1 H), 11.98 (s, 1 H), 8.86 (s, 1 H), 8.62 (d, \textit{J} = 3.60 Hz, 1 H), 8.54 (s, 1 H), 8.16 (d, \textit{J} = 11.60 Hz, 1 H), 8.02 (s, 1 H), 7.76 (t, \textit{J} = 10.00 Hz, 1 H), 7.61 (d, \textit{J} = 8.80 Hz, 1 H), 7.53-7.48 (m, 1 H), 2.55 (s, 3 H). \textsuperscript{13}C NMR (176 MHz, DMSO-\textsubscript{d\textsubscript{6}}) δ 164.2, 154.2, 151.0, 149.1, 144.7, 133.8, 130.9, 126.5, 124.4, 121.8, 117.1, 111.8, 15.2.

Synthesis of \textit{trans-3}: To a stirred solution of 2-methyl-1\textit{H}-benzo[\textit{d}]imidazole-6-carbohydrazide (300 mg, 1.577 mmol) in ethanol (5.0 mL), were added isonicotinaldehyde (0.154 mL, 1.577 mmol) and acetic acid (18.94 mg, 0.315 mmol) at 0 °C, and the reaction mixture was stirred at 80 °C for 16 hours. After completion of reaction, reaction mixture was evaporated under reduced pressure to obtain crude mixture. Then washed with MTBE (20.0 mL), followed by washings with ethanol (10.0 mL), and dried under reduced pressure to afford \textit{trans-3} (230 mg, 51.4 %) as an off-white solid. LCMS: Calculated for C\textsubscript{15}H\textsubscript{13}N\textsubscript{5}O, Molecular Weight: 279.30, Observed 280.2 (M+H), RT. 1.388 min, 99.74 % (Max), HPLC: 3.064 min, 98.53% (Max). \textsuperscript{1}H NMR (400 MHz, DMSO-\textsubscript{d\textsubscript{6}}): δ 12.62 (s, 1 H), 12.09 (s, 1 H), 8.67 (d, \textit{J} = 4.40 Hz, 2 H), 8.47 (s, 1 H), 8.12 (s, 1 H), 7.77 (d, \textit{J} = 8.40 Hz, 1 H), 7.68 (d, \textit{J} = 3.20 Hz, 2 H), 7.58 (d, \textit{J} = 8.40 Hz, 1 H), 2.54 (s, 3 H). \textsuperscript{13}C NMR (176 MHz, DMSO-\textsubscript{d\textsubscript{6}}) δ 162.0, 152.0, 148.4, 142.7, 139.9, 139.3, 137.1, 124.1, 119.6, 119.1, 112.8, 111.8, 12.9.
Table S3. Data summary for the 4 synthesized isomers of trans-1.

| Cpd. | MW (g/mol) | LogD (pH 7.4) | TPSA (Å²) | Kinetic solubility (µM) | Clna (h, m) | Kinetic solubility (µM) | CACO-2 (A>B 10e-6 cm/s; B->A 10e-6 cm/s; ER) | CSP [Hz] | relaxation |
|------|------------|---------------|-----------|------------------------|-------------|-------------------------|---------------------------------|---------|------------|
| trans-1 | 279.3 | 2.7 | 83 | ND | <10 ;15 | 0.44 ; 8.65 ; 19.8 | 1 | 35% |
| cis-12 | 279.3 | >3.8 | 83 | ND | 61 ; 688 | 0.83 ; 36.6 ; 43.3 | 4.8 | 60% |
| trans-2 | 279.3 | ND | 83 | ND | <10 ; 22 | 0.04 ; 6.23 ; 149 | 1.4 | 30% |
| trans-3 | 279.3 | 2.7 | 83 | >200 | ND | 0.04 ; 9.79 ; 238 | 2 | 25% |

LogD Determination: Octanol saturated Phosphate Buffer 20 mM pH 7.4 was prepared by placing 100 mL Sorensen Phosphate Buffer 0.2 M pH 7.4 (EMS, Cat.-No. 11601-40) in a 1000 mL volumetric flask and ultrapure water was added to volume. Then 20 mL Octanol (Merck, Cat.-No.: 100991) to the Buffer was added, stirred overnight, and the phases were separated. Buffer saturated Octanol was prepared by adding 20 mL buffer (EMD, Cat.-No. 11601-40, as described above) to 1 L of Octanol (Merck, Cat.-No.: 1.00991), stirred overnight and the phases were separated. Eight µL of 10 mM compound solution in DMSO was pipetted into a 0.5 mL safe-lock tube. Then 200 µL of the saturated Octanol and 200 µL of the saturated buffer solution were added. Overhead rotation was performed for 1 hour at RT. The sample was centrifuged for 2 min at 1000 rpm. 80 µL of the Octanol-phase (upper phase) was pipetted into an HPLC Vial and diluted with 720 µL methanol (dilution factor = 10). The buffer-phase (lower phase) will be taken with an insulin jab through the bottom of the safe-lock tube and injected in a HPLC (XBridge C8 3.5µm, 30 °C, UV 220, 254 and 290 nm, DAD detector). A suitable wavelength for evaluation of the chromatogram was selected to obtain maximum sensitivity to allow determination of high log D values (e.g., log D > 3.0). log D = log10 [ (area Octanol * dilution factor Octanol / Injection volume Octanol) / (area buffer / Injection volume buffer)].

pKa Determination: In fast UV titration, the shift in the UV spectrum brought about by the ionization of the compound during the titration is monitored. The pH at which 50% of the species are in the ionized form represents the pKa of the tested compound. Sirius T3® is a fully automated instrument that utilizes an Ag/AgCl combination pH electrode. The instrument can accurately detect the pKa in the pH range of 1.5 – 12.0. SiriusT3® is used for the determination of pKa of the analyte by fast UV titration. 5 µL of a 10 mM DMSO solution of the analyte is pipetted into a glass vial and Ionic Strength Adjusted (ISA) water (0.15 M KCl
solution) along with 80% v/v methanol are added. The solution is adjusted to pH 1.5 with 0.5 M hydrochloric acid (HCl) and titrated to pH 12.0 with 0.5 M potassium hydroxide (KOH) solution in the first titration. During the titration the solution is stirred with a built-in overhead stirrer and the spectral shift due to ionization as a factor of pH of the solution is monitored by the UV dip probe in the instrument. Prior to the second titration, the solution is again adjusted to pH 1.5 with 0.5 M hydrochloric acid (HCl) and an additional cosolvent is added to the solution. The solution is further titrated to pH 12.0 with 0.5 M KOH. Likewise, cosolvent is added to the solution, after adjustment of the pH to 1.5, prior to the third titration and the solution is titrated again to pH 12.0 with 0.5 M KOH. The Yasuda-Shedlovsky plot is used to calculate the pKa of the compound, by applying an appropriate correction factor for the amount of cosolvent in the medium.

Solubility measurement: 2.0 mg of compound was accurately weighed into a Uniprep® syringeless filter (5ml 0.45 µm), and 2 mL of solvent was added and shaken for 24 hours at 37°C. The pH was checked after 6 to 8 hours and if the pH deviated by more than 0.05 units, it was adjusted with 0.1 N HCl or 0.1 N NaOH solutions. The suspension was filtered after 24 hours and the concentration of dissolved substance was determined by HPLC after suitable dilutions with corresponding buffer if required. The result was expressed in µg/ml.

In vitro Metabolism in Liver Microsomes: The metabolic stability assay was performed in duplicate in a 96-well microtiter plate. The test compounds (0.1 µM) were incubated (37 °C) in mouse, rat and pooled human liver microsomes (final protein concentration of 0.4 mg/mL; XenoTech, Lenexa, KS) suspended in 0.1 M phosphate buffer (pH 7.4) for predetermined time points, in the presence and absence of the cofactor NADPH (1 mM). The reactions were quenched by the addition of ice-cold acetonitrile containing internal standard (carbamazepine, 0.0236 µg/mL). The samples were centrifuged and the supernatant was filtered and analyzed by means of LC-MS/MS (Agilent Rapid Resolution HPLC, AB SCIEX 4500 MS). The relative loss of parent compound over time was monitored and plots were prepared for each compound of concentration versus time to determine the first order rate constant for compound depletion.

Permeability across Caco-2 cell monolayers: Caco-2 cells were seeded onto 0.3 cm2 polycarbonate filter trans-wells at a density of 60,000 cells per well. The transport experiment was conducted using confluent cell monolayers on days 22-23 post-seeding. On the day of the permeability study, the integrity of the cell monolayers was determined by measuring the transepithelial electrical resistance (TEER) in the presence of pH 7.4 Hanks balanced salt solution, and only monolayers with TEER values of >400.cm² were selected for use in the study. Permeability experiments were performed using human plasma as the transport medium in both the apical and basolateral chambers to overcome issues associated with poor
mass balance of (-)-1 when using buffer as the transport medium. The permeability of marker compounds (i.e., $^{14}$C-mannitol, $^{3}$H-propranolol, and $^{3}$H-digoxin) was also assessed using the plasma transport medium with a subset of wells from the same batch to ensure assay performance. Donor solutions were prepared by spiking stock solutions of marker compounds into human plasma (final DMSO concentration of 0.1% v/v) at a nominal concentration of 20 μM. The solution was equilibrated at 37 °C for 4 hours before centrifuging for 5 minutes to remove any compound that may have precipitated. The visually clear supernatant plasma was then added to the transport wells. Samples from the donor chamber were taken at the start (within 2 minutes) and end of the experiment. Compound flux was determined over a period of 240 minutes with samples taken from the acceptor chamber at 6-9 time points. At each sample time, the volume of acceptor plasma removed was replaced with blank plasma. Acceptor concentrations were then corrected for the dilution that occurred with plasma replacement. Donor and acceptor samples were stored frozen at -80 °C until analysis by LC-MS. The measured initial donor concentration (CD) was corrected for the free concentration using the measured fraction unbound (fu) and the apparent permeability coefficient (Papp) was calculated using the following equation:

$$P_{app} = \frac{dQ/dt}{CD \times fu} \times \frac{1}{A}$$

where $dQ/dt$ is the apparent steady-state flux and A is the monolayer surface area. The efflux ratio was calculated as the ratio B-A Papp/A-B Papp.

**S. mansoni adult worm assays**

Animal experiments were carried out following Swiss national and cantonal regulations on animal welfare at the Swiss Tropical and Public Health Institute (Basel, Switzerland) under the permission number 2070. Adult *S. mansoni* worms were collected by dissecting the mesenteric veins of infected mice after day 49 post-infection. 6 worms were placed in 2 ml supplemented RPMI medium (5% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin) at 37 °C and 5% CO$_2$ in the presence of 0.2-20 µM of the test compounds. 96 h post-incubation 1 ml of sample was taken from the wells, centrifuged first at 2000g for 5 min at 4°C, followed by 14.000g for 20 min at 4°C and the supernatant stored at -80°C. Control samples included DMSO only.

**miRNA analysis from S. mansoni worm secretome**

**Sample preparation and total RNA extraction**

Total RNA was extracted either from cell culture supernatant or frozen worms using the miRNeasy Mini Kit (Qiagen, Germany). For cell culture supernatants 800 μL of conditioned media were concentrated with Amicon 10kDa filter tubes to 200 μL. For each sample, 200 μL
concentrated media were homogenized with 1000 µL Qiazol. Frozen worms were mixed with 700µL Qiazol and homogenized using the TissueRuptor (Qiagen) for 30s at full speed. A synthetic RNA oligonucleotide mix obtained from the miRCURY Spike-In kit (Qiagen, Germany) was added to each sample after homogenization at equimolar amounts prior to RNA extraction. These spike-ins were subsequently used to monitor RNA extraction efficiency. Following incubation at room temperature for 10 minutes, chloroform was added to the lysates followed by cooled centrifugation at 12,000 G for 15 minutes at 4°C. For cell culture samples precisely 650 µL and for worm samples 450 µL of the upper aqueous phase were mixed with glycogen to a final concentration of 50 µg/mL to enhance precipitation. Samples were transferred to a miRNeasy mini column, and RNA was precipitated with 1.5 volumes of ethanol followed by automated washing with RPE and RWT buffer in a QiaCube liquid handling robot (Qiagen, Germany). Finally, total RNA was eluted in 30 µL nuclease free water and stored at -80°C until further analysis.

miRNA RT-qPCR analysis

Starting from total RNA samples, cDNA was synthesized using the miRCURY RT Kit (Qiagen, Germany). Reaction conditions were set according to recommendations by the manufacturer. For analysis of total RNA from worm samples 20 ng were used as input. For cell culture samples RNA concentrations were below the limit of quantification. Therefore, 2 µL total RNA were used as input in a 10 µL RT reaction. To monitor RT efficiency and presence of impurities with inhibitory activity, a synthetic DNA spike-in (cel-miR-39-3p) was added to the RT reaction. Quantitative PCR reactions were set up using miRCURY SYBR® green master mix and commercial LNA-enhanced primer assay for sma-miR-10-5p, sma-miR-125a-5p and sma-bantam-3p. Reactions were performed in a 96-well plate format in a Roche LC96 instrument (Roche, Germany) with the following temperature settings: 95°C for 10 min, 60 cycles of 95°C for 10 s and 60°C for 60 s, followed by melting curve analysis. Cq-values from cell culture samples from were normalized to RNA-spike in control level and worm sample to cDNA Spike-in control by subtracting the individual miRNA Cq-value from Spike-In Cq, thus obtaining a delta-Cq.
Figure S3: miR-10-5p quantification in worms (A) and culture supernatant („secreted“, B) after 96 hours. Relative change in sma-miR-10-5p levels compared to control average (n=2) are shown for three different concentrations (n=3). Adjusted p-values obtained from a Kruskal-Wallis Test in combination with Dunn’s multiple comparison test are shown.
Figure S4: Cellular assays to investigate phenotypic effect of remaining compounds on sma-miR-10 activity. As in Figure 4, NF-κB luciferase reporter Jurkat cells were DOTAP-transfected with 0.25µM duplexed sma-miR-10, or exposed to 5-10 Schistosoma mansoni worms via a 0.4µm transwell. Both transfection and worm exposure led to a 50% reduction in luciferase and junb expression, which was rescued in a dose-dependent manner by each of the compounds. (A-C) cis1, (D-F) 7A, (G-I) 8A. Mean + SEM; Multiple unpaired t tests, * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001. C, F, I, n = 2. All others n=3.