Supplementary Information

Potent Acridone Antimalarial against All Three Life Stages of *Plasmodium*

Papireddy Kancharla[1,], Rozalia A. Dodean[1,2], Yuexin Li[2], Xiaowei Zhang[2], Sean Kelly[2], Jordan Charlton[3], Angley Binauhan[3], Laurize Garcia[3], Diana Caridha[4], Michael S. Madejczyk[4], Xiannu Jin[4], William E. Dennis[4], Karl Kudyba[4], Sharon McEnearney[4], Partricia J. Lee[4], Cameron Blount[4], Jesse DeLuca[4], Chua Vuong[4], Kristina Pannone[4], Hieu T. Dinh[4], Kennedy Mdaki[4], Susan Leed[4], Monica L. Martin[4], Brandon S. Pybus[4], Anongruk Chimong[5], Liwang Cui[5], Joel Vega-Rodriguez[6], Martin Okitwi[7], Oriana Kreutzfeld[8], Ying Liu[9], Christina Docan[9], Carol Green[9], Aaron Agulay[9], Jon Mirsalis[9], Sarah N. Farrell[10], Geoffrey I. McFadden[10], Christopher D. Goodman[10], Alexandra S. Probst[11], Aaron Nilsen[2,12], Flaminia Catteruccia[11], Philip J. Rosenthal[8], Michael Riscoe[2,12], Roland Cooper[3], Alison Roth[3], and Jane Kelly[1,2,*]*

[1]Department of Chemistry, Portland State University, Portland, Oregon 97201, United States

[2]Department of Veterans Affairs Medical Center, Portland, Oregon 97239, United States

[3]Department of Natural Sciences and Mathematics, Dominican University of California, San Rafael, California 94901, United States

[4]Experimental Therapeutics Branch, Walter Reed Army Institute of Research, Silver Spring, Maryland 20910, United States

[5]Department of Internal Medicine, Morsani College of Medicine, University of South Florida, Tampa, Florida 33612, United States

[6]Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institute of Health, Rockville, Maryland 20852, United States

[7]Infectious Diseases Research Collaboration, Kampala, Uganda

[8]Department of Medicine, University of California San Francisco, San Francisco, California 94143, United States

[9]SRI Biosciences, SRI International, Menlo Park, California 94025, United States

[10]School of Biosciences, The University of Melbourne, Parkville, VIC, 3010, Australia

[11]Department of Immunology and Infectious Diseases, Harvard TH Chan School of Public Health, Boston, Massachusetts, 02115, United States

[12]Department of Microbiology and Molecular Immunology, Oregon Health & Science University, Portland, Oregon 97239, United States

Corresponding Author(s) Mail Address

*Papireddy Kancharla: 1719 SW 10th Avenue, Science Research and Teaching Center, Room Number 306, Portland, Oregon 97201, United States. Email: papiredd@pdx.edu

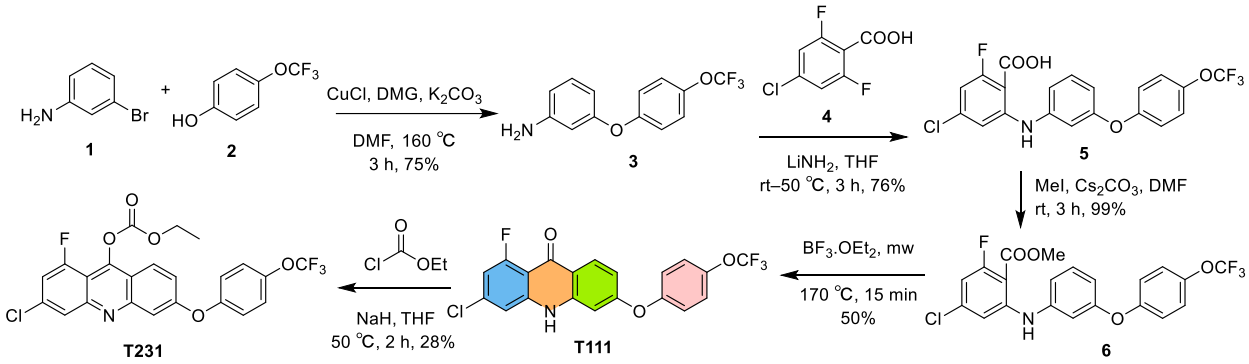
*Jane X. Kelly: 1719 SW 10th Avenue, Science Research and Teaching Center, Room Number 262, Portland, Oregon 97201, United States. Email: kellyja@ohsu.edu

Table of Contents

GENERAL REMARKS.

NMR spectra were recorded on Bruker AMX-400 spectrometer at 400 MHz. NMR experiments were recorded in $CDCl_3$ and DMSO-d_6 at 25 ℃. Chemical shifts (δ) are given in parts per million (ppm) downfield from internal standard tetramethylsilane (TMS), and coupling constants (J) are expressed in Hertz (Hz). High-resolution mass spectra (HRMS) (electrospray ionization (ESI)) were recorded on a vanquish UHPLC/HPLC system coupled with a high resolution (35000) Q Exactive Orbitrap mass spectrometer. GC-MS was performed using an Agilent Technologies 7890B gas chromatograph (30 m, DBS column set at either 100 or 200 ℃ for 2 min then at 30 ℃/min ramp to 300 ℃ with inlet temperature set at 250 ℃) using an Agilent Technologies 5977A mass-selective detector operating at 70 eV. Flash chromatography on silica gel was performed on either Isolera One flash chromatography system from Biotage or CombiFlash instruments with hexanes and ethyl acetate as eluents. Unless otherwise stated, all reagents and solvents were purchased from commercial suppliers and used without further purification. Reactions that

required anhydrous conditions were carried out under an atmosphere of argon. The microwave reactions were conducted using Biotage® Initiator+ microwave synthesizer. Analytical HPLC analysis was performed on an Agilent 1260 Infinity II LC System using C8 column (2.1 × 50 mm) with a linear elution gradient of water/methanol (containing 10 mM ammonium acetate) ranging from 50:50 to 0:100 for 10 min at a flow rate of 0.5 mL/min, at 254 nm. A purity of >95% has been established for T111and T231.



Scheme S1. Synthesis of acridone analogue T111 and its prodrug T231.

CHEMISTRY EXPERIMENTAL PROCEDURES

Synthesis of 3-(4-(Trifluoromethoxy)phenoxy)aniline (3). A mixture of copper (I) chloride (CuCl) (5.76 g, 58.2 mmol) and *N*,*N*-dimethylglycine (DMG) (3.99 g, 38.7 mmol) in 100 mL of dimethylformamide (DMF) was stirred under vacuum at 50 °C for 15 min. To the blue-colored catalyst mixture were added anhydrous K_2CO_3 (100.4 g, 727 mmol), *m*-bromoaniline (**1**) (50.0 g, 291 mmol), 4-(trifluoromethoxy)phenol (**2**) (51.7 g, 291 mmol), and 500 mL of DMF. The reaction mixture was degassed again at 50 °C for 20 min, purged with argon, then stirred at 160 °C for 3h. Upon cooling to room temperature, the reaction mixture was filtered through a pad of silica gel and washed with ethyl acetate. The filtrate was concentrated by rotary evaporator and the crude product was chromatographed on silica gel, with hexanes/ethyl acetate as eluent, to afford the title

compound **3** as a light brown oil (59.0 g, 75%). The identity of **3** was further conformed by GC-MS analysis and characterized by using NMR and HRMS spectral data. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.14–7.11 (m, 4H), 6.87–6.84 (m, 4H), 5.54 (brs, 2H).; HRMS (ESI) calcd for $C_{13}H_{11}F_3NO_2$ $[M + H]^+$ 270.0736; Found 270.0733.

Synthesis of 4-Chloro-2-fluoro-6-((3-(4-(trifluoromethoxy)phenoxy)phenyl)amino)benzoic acid (5). A solution of $LiNH_2$ (22.0 g, 959 mmol) in 300 mL anhydrous tetrahydrofuran (THF) was stirred for 15 min at room temperature, followed by slow addition of a slurry of **3** (73.7 g, 274 mmol) and 4-chloro-2,6-difluorobenzoic acid (**4**) (52.8 g, 274 mmol) in THF (200 mL). The mixture was stirred for 1.5 h at 50 °C, when sudden bubbling was observed and the slurry turned into a clear solution. After an additional 1.5 h the completion of the reaction was confirmed by GC-MS analysis. Acetonitrile (100 mL) was added to the reaction mixture and stirred for 1 h, then the solvents were removed under reduced pressure. Addition of 200 mL ethanol and 200 mL water, followed by acidification with 2 N HCl yielded a precipitate that was filtered and washed with water. The crude solid material was chromatographed on silica gel, with hexanes/ethyl acetate as eluent, to afford the title compound **5** as a beige solid (92.2 g, 76%). The identity of **5** was further conformed by GC-MS analysis and characterized using NMR and HRMS spectral data. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.17 (s, 1H), 7.41–7.37 (m, 3H), 7.19–7.17 (m, 2H), 7.03 (ddd, J = 8.1, 2.1, 0.8 Hz, 1H), 6.96 (dd, J = 1.9, 1.3 Hz, 1H), 6.89 (t, J = 2.2 Hz, 1H), 6.87–6.83 (m, 1H), 6.78 (ddd, J = 8.2, 2.4, 0.8 Hz, 1H).; HRMS (ESI) calcd for $C_{20}H_{13}ClF_4NO_4$ $[M + H]^+$ 442.0464; Found 442.0464.

Synthesis of Methyl 4-Chloro-2-fluoro-6-((3-(4-(trifluoromethoxy)phenoxy)phenyl)amino)benzoate (6). To a stirred solution of **5** (41.0 g, 92.8 mmol) in DMF (300 mL) was added Cs_2CO_3 (16.6 g, 51.0 mmol), followed by slow addition of

methyl iodide (13.8 g, 97.4 mmol). The mixture was stirred at room temperature for 3 h, and the consumption of **5** was confirmed by GC-MS analysis. The solid residue was then filtered off, and washed with methanol. The filtrate was concentrated under reduced pressure to afford the crude product, which was chromatographed on silica gel with hexanes/ethyl acetate as eluent, to yield **6** as a white solid (41.8 g, 99%). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.82 (s, 1H), 7.41–7.36 (m, 3H), 7.19–7.17 (m, 2H), 7.01–6.97 (m, 2H), 6.90 (dd, J = 10.5, 1.8 Hz, 1H), 6.83 (t, J = 2.2 Hz, 1H), 6.77 (ddd, J = 8.2, 2.4, 0.8 Hz, 1H), 3.80 (s, 3H).; HRMS (ESI) calcd for $C_{21}H_{15}ClF_4NO_4$ $[M + H]^+$ 456.0620; Found 456.0613

Synthesis of Acridone Analog T111. To a microwave reaction vial were added **6** (6.00 g, 13.2 mmol) and $BF_3.Et_2O$ (8.14 mL, 65.9 mmol), and the mixture was exposed to microwave radiation for 15 min at 170 ℃. After cooling to room temperature, the reaction mixture was poured into water (100 mL) and allowed to stir for 5 min. The solid material was filtered by a sintered funnel and washed with water, then dried. Recrystallization from DMF/ethanol (3:1) yielded T111 as a white solid (3.23 g, 50%). Due to the limited volume capacity of the microwave reaction vials, we conducted multiple 6.00 g scale batches to generate over 50 g of the T111. ^{1}H NMR (400 MHz; DMSO-d_6): δ 11.74 (s, 1H), 8.16 (d, J = 8.9 Hz, 1H), 7.52 (d, J = 8.4 Hz, 2H), 7.37 (d, J = 8.7 Hz, 2H), 7.21 (s, 1H), 7.09 (dd, J = 11.5, 1.9 Hz, 1H), 7.00 (dd, J = 8.6, 1.1 Hz, 1H), 6.75 (d, J = 1.0 Hz, 1H); HRMS (ESI) calcd for $C_{20}H_{11}ClF_4NO_3$ $[M + H]^+$ 424.0358; Found 424.0350.

Synthesis of Prodrug T231. A slurry of T111 (1.27 g, 3.00 mmol) in 50 mL of THF was added NaH (0.215 g, 9.00 mmol) under argon, and stirred at 50 ℃ for 15 min. To the reaction mixture was added ethyl chloroformate (0.650 g, 6.00 mmol), and stirred at 50 ℃ for 2 h. After quenching with isopropanol and methanol, the crude reaction mixture was concentrated, and flash chromatography with hexanes/ethyl acetate as eluent yielded T231 as a white solid (0.413 g, 28%).

^{1}H NMR (400 MHz; DMSO-d_6): δ 8.31 (d, J = 9.5 Hz, 1H), 8.05 (s, 1H), 7.69–7.62 (m, 2H), 7.55 (d, J = 8.5 Hz, 2H), 7.44 (d, J = 9.1 Hz, 2H), 7.30 (d, J = 2.2 Hz, 1H), 4.42 (q, J = 7.1 Hz, 2H), 1.38 (t, J = 7.1 Hz, 3H); HRMS (ESI) calcd for $C_{23}H_{15}ClF_4NO_5$ $[M + H]^+$ 496.0569; Found 496.0573.

BIOLOGICAL EXPERIMENTS

In Vitro Drug Susceptibility Assay Against ART-Resistant Parasites. Synchronized ring stage parasites were cultured for 72 h in the presence of test drug. The assay was performed in 96-well plates at 2% hematocrit and 0.5% starting parasitemia. Each experiment was conducted in triplicate and repeated with three biological replicates. Parasite growth at 72 h was quantified via SYBR Green I staining. The obtained counts were plotted against the logarithm of the compound concentration and fitted a curve with nonlinear regression (sigmoidal dose-response/variable slope equation) to determine IC_{50} values. The data was analyzed using GraphPad Prism software 8.0.

Ex Vivo Drug Susceptibility Assay Against Clinical Isolates. For parasite culture, parasitemia was identified with Giemsa-stained thin films using a light microscope. Samples containing only a minimum of 0·3% parasitemia were analyzed. Isolates were stored at 4 ℃ and assayed within 24 h of collection. To measure ex vivo drug susceptibilities, a 72-h microplate growth inhibition assay was used with SYBR Green detection. Study compounds were dissolved in dimethyl sulfoxide as 10 mM stocks and stored at –20 ℃. Drugs were serially diluted, including drug-free and parasite-free control wells, with concentrations optimized to capture full dose-response curves. Cultures were diluted with uninfected O+ erythrocytes to achieve 0.2% parasitemia and 2% hematocrit. After 72 h, wells were resuspended and culture from each well was transferred to 96-well plates containing SYBR Green lysis buffer and mixed. Plates were incubated for 1 h in the dark at room temperature and fluorescence was measured with a plate reader (485 nm excitation

and 530 nm emission). IC_{50} values were derived by plotting fluorescence intensity against log drug concentration and fit to a non-linear curve using a four-parameter Hill equation in Prism (version 9.0).

In Vivo Antimalarial Efficacy Against Blood Stage *P. Yoelii* in Rodent Model. Female CF1 mice (4-5 weeks old) were infected intravenously with 2.5×10^5 *P. yoelii* (Kenya strain) from a donor animal. Drug administration commenced the day after the inoculation (day 1). Test drug or the drug combination was dissolved in PEG-400 and administered by oral gavage once daily for four successive days in the 4-day suppression model but only once on day 1 in the single dose cure model. Blood was obtained on day 5 and then at weekly intervals through day 28. Blood films were Giemsa-stained and examined microscopically to determine parasitemia. All mice were observed daily to assess clinical signs. Animals with observable parasitemia following the experiment were euthanized and animals cleared of parasites were observed daily, with assessment of parasitemia performed weekly until day 28, at which point the animals were scored as cured of infection. ED_{50} and ED_{90} values were derived graphically from the dose required to reduce the parasite burden by 50% and 90%, respectively, relative to drug-free controls.

In Vitro Antiplasmodial Activity Against Sexual Blood Stage *P. falciparum* Gametocytes. *P. falciparum* NF54 parasites were cultured in O+ red blood cells using RPMI 1640 medium, which was supplemented with 25 mM HEPES, 50 mg/L hypoxanthine, 25 mM NaHCO3, and 10% (vol/vol) heat-inactivated type O+ human serum (Interstate Blood Bank, Inc.). The cultures were maintained at 37°C in a gas mixture of 5% O2, 5% CO2, and balanced N2. On days 5 to 7 post-induction, the cultures were treated with either 0.1 or 10 μM of T111, diluted in DMSO. The control group was treated with DMSO alone. Gametocytemia was assessed on day 14 post-induction using Giemsa-stained smears and microscopy.

In Vitro Liver Stage *P. Cynomolgi* Assay. Cryopreserved primary simian hepatocytes were obtained from BioIVT Inc. Sporozoites were dissected from *Anopheles* mosquitoes infected with *P. cynomolgi bastinaenllii* (B strain). Hepatocytes were seeded in 384-well plates on day -1 or -2, with sporozoite infection occurring on day 0. Test drugs were dissolved in DMSO and used in 2-fold 12-point serial dilutions. To study prophylaxis, drug exposure began 1 h post sporozoite infection on day 0, and continued on day 1 and 2. To study radical cure, drugs were administered on day 4, 5, 6, and 7 post sporozoite infection. Readout for both assays was on day 8 via immunofluorescence staining and imaging using an Operetta CLS high-content imager. Compound toxicity was measured in parallel by photometric analysis of host hepatocyte viability. Hypnozoite or schizont detection and classification were performed using Harmony 4.9 software. IC_{50}s were generated based by fitting a 4-parameter logistic curve via python adaptation and grid algorithm.

In Vivo Antimalarial Efficacy Against Liver Stage *P. Berghei* in Rodent Model. Luciferase-expressing *P. berghei* were extracted from the salivary glands of *Anopheles* mosquitoes. C57BL/6 male albino mice were infected intravenously with 10,000 sporozoites on day 0. Drugs (dissolved in PEG-400) were administered orally on days -1, 0, and +1. The parasite load in the liver and blood (approximately at 24, 48, and 72 h post infection) was assessed using an IVIS spectrum instrument. IVIS Living Image software was used to quantify bioluminescence from the liver region or whole animal surface area for assessments of liver-stage and blood-stage efficacy. Starting on day 6 post infection, all mice were assessed for blood stage parasitemia, which was quantitated from blood using flow cytometry conducted using a FC500 MPL flow cytometer. Parasitemia was monitored every week for up to 31 days post-infection. Mice tested negative for parasitemia on day 31 were considered cured.

Standard Membrane Feeding Assay (SMFA) in Mosquitoes. Deidentified human blood and serum were obtained from the Australian Red Cross Blood Service. Mature (stage V) gametocytes were quantified in Giemsa-stained blood smears. Immediately prior to infection, a feeding mixture comprising 45% human serum and 55% human blood containing 0.15% stage V gametocytes and the desired concentration of drug or vehicle control, was prepared and maintained at 37 ℃. Adult female *Anopheles stephensi* (MR4) mosquitoes reared under the standard insectary conditions were placed in mesh-topped cups and allowed to feed on the gametocyte mixtures in prewarmed glass feeders wrapped with parafilm. Mosquitoes that failed to feed in 20 min were removed. Mosquito midguts were dissected 7 days after infection, stained in 0.2% mercurochrome, and oocysts were counted using a light microscope.

Tarsal Contact Assay in Mosquitoes.

Preparation of Thin Film. For preparation of the thin film, 25 mL xylenes and 1.0 g low density polyethylene polymer were combined on the lid of a glass petri dish (6 cm) and heated at 150 ℃ until the polymer was dissolved. Xylenes were occasionally replenished upon evaporation. A solution of xylenes containing T111 prodrug was then added to the above solution. Excess xylenes were evaporated slowly and the resulting crude polymer films were allowed to dry overnight. The crude polymer thin film was then put underneath the glass petri dish, and the assembled apparatus was heated on a hotplate with a weight placed on top. The resulting extruded polymer thin film containing the 5% (w/w) T111 prodrug in low-density polyethylene (LDPE) was allowed to cool and removed from the petri dish lid.

Tarsal Contact Assay. The T111 prodrug film was placed in a 6 cm glass petri dish fitted with a transparent lid to contain mosquitoes. 4–7-day old female *Anopheles gambiae* mosquitoes were aspirated into this container and allowed to land on the T111 prodrug thin film for six minutes.

After exposure, the mosquitoes were immediately transferred to a cage and one hour later provided with cultured *P. falciparum* NF54 gametocyte-infected erythrocytes using a membrane feeder. Infected mosquitoes were housed in a secure infection glovebox for the remainder of the experiments and provided with 10% w/v glucose water solution *ad libitum*. On day 7 post-infection, mosquitoes were aspirated into 80% ethanol and incubated at -20 °C for ten minutes before being transferred to 1X PBS for dissection. Midguts were dissected into 1X PBS, stained with 0.2% w/v mercurochrome (mercury dibromofluorescein disodium salt, Sigma-Aldrich) in ddH_2O for 14 minutes, then mounted in 0.02% mercurochrome for microscopy. Midguts were imaged and oocysts counted at 100X magnification on an Olympus Inverted CKX41 microscope to quantify oocyst prevalence and intensity.

Synergy Analysis for Drug Combination Studies.

In Vitro Drug Combination Studies Against Blood Stage *P. Falciparum* and Liver Stage *P. Cynomolgi*. Drugs were diluted in fixed ratios (4:1, 3:2, 2:3 and 1:4) of starting concentrations predetermined to generate well-defined concentration response curves. Intrinsic dose-response curve for each drug alone and four different fixed-ratio combination dose-response curves yielded corresponding IC_{50}s. The fractional inhibitory concentrations (FICs) were then calculated by the following formulas: FIC (A) = IC_{50} of drug A in combination/IC50 of drug A alone; FIC (B) = IC_{50} of drug B in combination/IC_{50} of drug B alone; FIC index = FIC (A) + FIC (B). The isobolograms were constructed by plotting a pair of FICs for each combination. An interpretation of a straight diagonal line (FIC index = 1) on the isobologram indicates an additive effect between the two drugs. A concave curve below the line (FIC index < 1.0) indicates synergy of the combination, while a convex curve above the line (FIC index > 1.0) indicates antagonism.

Ames Test. Tester strain *S. typhimurium* (TA100 and TA98) cultures were inoculated from the lyophilized pellets and grown in nutrient broth provided by EBPI. Each inoculated vial was placed overnight in the incubator at 37 °C. The positive controls for the assay were 2-aminoanthracene (2-AA) for incubations with an S9 mix and sodium azide (NaN_3, used for TA100) and 2-nitrofluorene (2NF, used for TA98) for incubations without the S9 mix. The tester strains were mixed with reaction mixture and, where appropriate, S9 mixture, test compounds were then added to each reaction tube. An aliquot of the mixture was then added to each well of a 96-well plate. The 96-well plates were covered and sealed in zip-lock bags and incubated for 5 days at 37 °C. Plates were scored visually, and the positive wells against background mutations were statistically analyzed.

Eryptosis Assay. Fresh Li-heparin-anticoagulated blood samples were obtained from the Clinical Trial Center at WRAIR. The blood was centrifuged and the platelet and leukocyte-containing supernatant was removed. Erythrocytes were incubated with TQ or T111 in Ringer solution at 37 °C for 48 h. Flow cytometry was employed, to estimate stimulators of eryptosis: cell volume from forward scatter, reactive oxygen species (ROS) formation from 2′,7′-dichlorodihydrofluorescein (DCFDA) dependent fluorescence, and phosphatidylserine exposure at the cell surface from annexin-V-binding. Annexin-V is a small protein that has a high affinity for phosphatidylserine and annexin-V binding is used to measure the presence of the phosphatidylserine on the cell membrane surface and subsequently the eryptotic activity of the cell.

Annexin-V-Binding Assay for Detection of Phosphatidylserine Translocation. After incubation under the respective experimental condition, cell suspension was washed in Ringer solution containing $CaCl_2$ and then stained with Annexin-V-FITC at 37 °C for 15 min under protection from light. The annexin-V-abundance at the erythrocyte surface was subsequently

determined on a FACS Calibur. Annexin-V-binding was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. A marker (M1) was placed to set an arbitrary threshold between annexin-V-binding cells and control cells. The same threshold was used for untreated and drug treated erythrocytes.

Reactive Oxygen Species Detection Assay. Oxidative stress was determined utilizing 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 150 µL suspension of erythrocytes was washed in Ringer solution and stained with DCFDA in Ringer solution containing DCFDA at a final concentration of 10 µM. Erythrocytes were incubated at 37 °C for 30 min in the dark and washed two times in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in Ringer solution and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur. Subsequently, the geomean of the DCFDA dependent fluorescence was determined.

Forward Scatter Analysis for Volume of RBC. A dot plot of forward scatter (FSC) vs. side scatter (SSC) was set to linear scale for both parameters. The effect of testing compounds on RBC volume was analyzed on LSR Fortessa flow cytometer.

In Vivo Toxicology and Toxicokinetic Studies in Rats. T111 was dissolved in PEG-400 and continuously stirred in a heated water bath between 85 – 98 °C for 1-4 h, until a clear solution or a homogeneous formulation was obtained. For the MTD study, each group of two male and two female Sprague Dawley rats were administered a single dose of 30, 100, 200 or 400 mg/kg T111 by oral gavage and the animals were monitored immediately post dose, 2-4 h and 24 h post dose for clinical signs, with body weights recorded pre-dose and on Day 1 for each dose level group. For the 7-day repeat dose study, each group of eight male and eight female Sprague Dawley rats were administered a single oral dose of T111 (25, 100 or 400 mg/kg) or vehicle control daily for

7 consecutive days and evaluated for body weight changes (pre-dose, on day 8 and 14, and prior to euthanasia), clinical observations (immediately, 2-4 h post dose, and daily on day 1 to 7), plasma drug levels (on day 1 and 7), clinical pathology (hematology, clinical chemistry on Day 8 and 14), organ weights (brain, heart, kidney, liver, spleen; on Day 8 and 14), and gross and microscopic examination of the selected tissues (brain, cecum, colon, duodenum, heart, ileum, jejunum, kidneys, liver, lungs, lymph nodes, rectum, spleen, forestomach, glandular stomach; on day 8 and 14).

For toxicokinetic studies, blood was collected from animals at 6 time points post dose on Day 1 (0.5, 1, 2, 4, 8, and 24 h) and on Day 7 (0.5, 2, 8, 24, 48, and 96 h). Drug levels were determined in collected plasma samples using bioanalytical method developed at SRI. The plasma drug level data were analyzed using Phoenix WinNon (version 8.3) software to perform noncompartmental data analysis.

Drug Resistance Selection Studies. Four independent cultures of *P. falciparum* Dd2 were maintained in the presence of T111 (0.1 nM, 0.5 nM or 2 nM), with a non-drug exposed control grown in parallel. The concentration of T111 was incrementally increased when parasites replicated at the same rate as the untreated parent line for three life cycles. Selection experiments were completed when T111 pressure reached 1 μM, at which point the culture was cryopreserved. Cryopreserved parasites from specific T111 concentrations were thawed and cloned by limiting dilution. DNA extractions were performed on clonal parasites using the QIAamp DNA extraction kit (QIAGEN Inc, Valencia, CA) or ZYMO Quick gDNA Miniprep kit (Zymo Research Corp, Irvine, CA). For sequencing of the open reading frame of *pfcytb*, PCR was performed with genomic DNA using forward and reverse flanking primers for cyt b primer 1: 5'-TTCCTGATTATCCAGACGCT-3' and primer 2: 5'-TGTTCCGCTCAATACTCAGA-3'. PCR products were subjected to enzymatic cleanup with ExoSAP-IT (Thermo Fisher Scientific) and

sent for Sanger sequencing (Eurofins Genomics, Louisville, KY) with PCR primer 2 paired with an internal forward primer: 5'-GAGTTATTGGGGTGCAACTG-3' and an internal reverse primer: 5'-CACTCACAGTATATCCTCCACA-3'. Sequences were assembled using 4Peaks software (Nucleobytes, Amsterdam, Netherlands) and aligned with Clustal Omega (EMBL-EBI). For sequencing of the open reading frame of *pfdhodh*, PCR was performed with genomic DNA using forward primer: 5'-GTGATAGATAGCTCCAGTCGATTTC-3' and reverse primer: 5'-TTTGCGCACTTATGTGTCGCCC-3'. PCR products were prepared for Sanger sequencing and analyzed as described above, except using two forward primers: 5'-GCTATTAATGTAAGCTCCC-3' and 5'-CCATTCGGTGTTGCTGC-3' and with two reverse primers: 5'-GCATTACCCGTTTGGCCCCTTGGGG-3' and 5'-GGAGCTTACATTAATAG-3'.

Table S1. In Vitro hERG Inhibitory Activity of T111.

compound	concentration (μM)	%inhibition[a,b]
T111	3.125	2.81
	6.25	5.29
	12.5	3.73
	25	6.91
	50	1.87
	100	2.37
verapamil	0.01	7.85
	0.03	10.5
	0.1	26.3
	0.3	46.8
	1	69.8
	3	91.8
0.33% DMSO	-	6.75

[a] This assay was performed on the hERG-expressing CHO-K1 cell line;
[b]% Inhibition is the average of two replicates.

Table S2. Activity of Standard Antimalarials against *P. falciparum* T111 Mutant Lines.

Parasite Line	**Standard Antimalarial Drug**			
	CQ	**PIP**	**LUM**	**DHA**
Dd2	180 ± 17	7.4 ± 0.6	1.2 ± 0.1	1.7 ± 0.1

Dd2-A$^{131S\text{-}140I\text{-}259L\text{-}264L}$	205 ± 21	9.9 ± 0.6	1.7 ± 0.09[d]	2.3 ± 0.2
Dd2-B$^{131S\text{-}140I\text{-}259L\text{-}264L}$	168 ± 31	12 ± 2.3[d]	1.7 ± 0.4	2.3 ± 0.2
Dd2-D$_0^{119L\text{-}131S\text{-}259L}$‡	210 ± 34	11 ± 1.2 [d]	1.5 ± 0.1	2.2 ± 0.2
Dd2-D$_1^{131S\text{-}140I\text{-}259L}$‡	162 ± 18	9.1 ± 1.7	2.4 ± 0.1[a]	2.4 ± 0.2

Mean IC_{50} values ± SEM (standard error of mean) in nM; n ≥ 3.
One-way ANOVA test followed by a Dunnett's multiple comparison test of mean IC_{50} values of mutants vs Dd2 parent line where: $p < 0.0001$ [a], $p < 0.001$ [b], $p < 0.01$ [c], $p < 0.05$ [d].
‡ = Parasite lines harboring a mutation in *pfdhodh*
CQ: chloroquine; PIP: piperaquine; LUM: lumefantrine; DHA: dihydroartemisinin

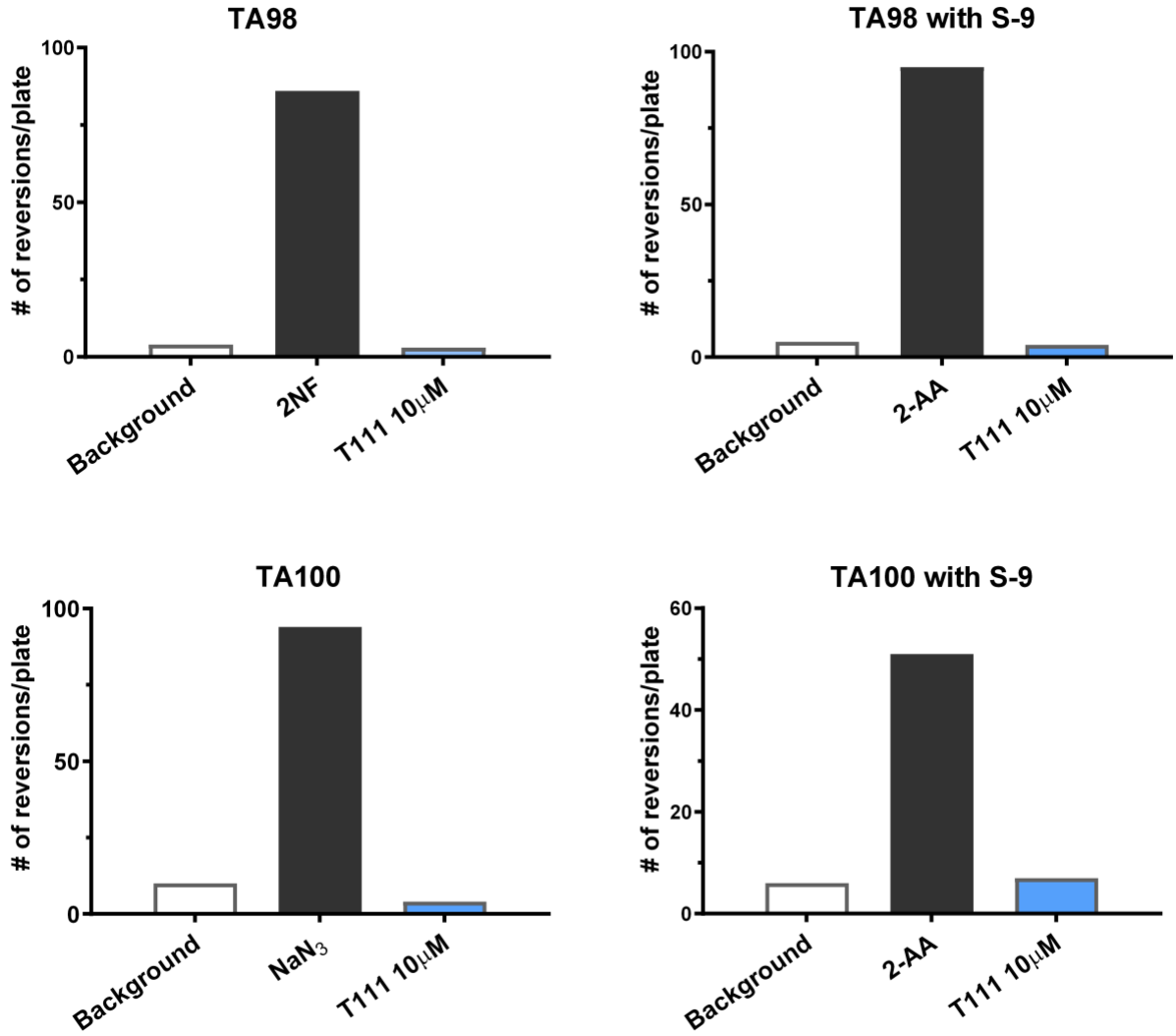


Figure S1. In vitro mutagenicity screening of T111 at 10 μM concentration and positive controls (2NF, 2-nitrofluoren; 2-AA, 2-aminoantheacene; NaN_3, sodium azide).

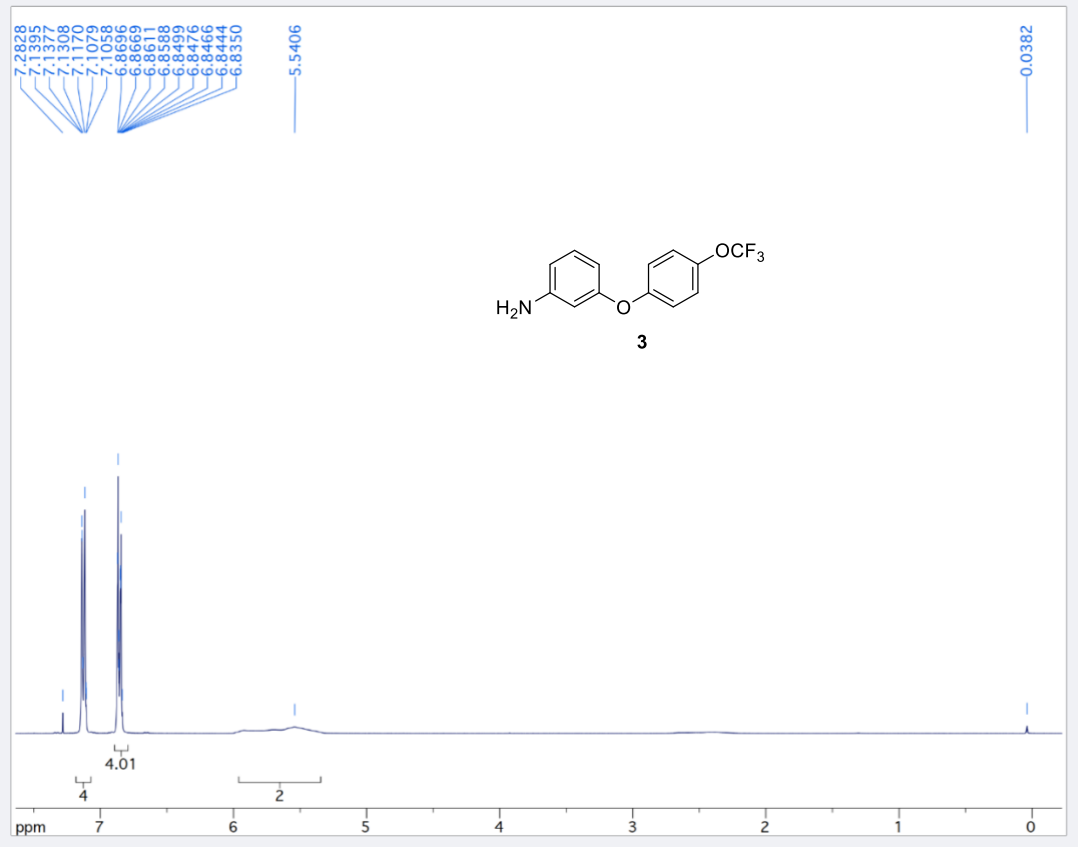


Figure S2. ^{1}H NMR spectrum of intermediate **3** ($CDCl_3$, 400 MHz).

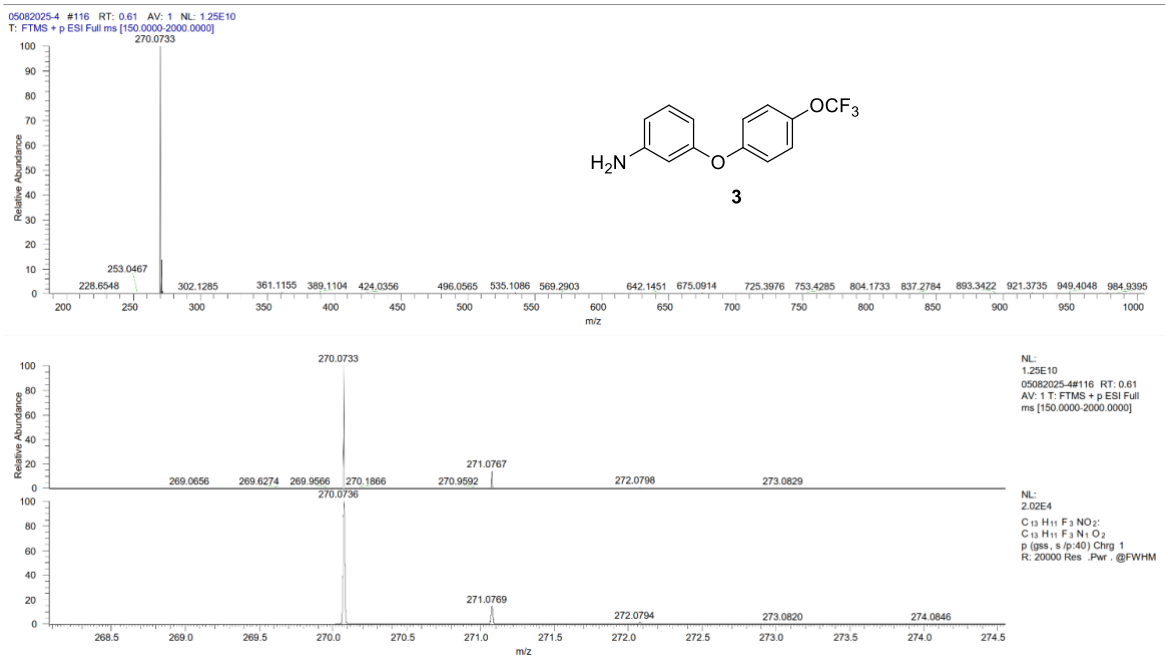


Figure S3. HRMS (ESI) of intermediate **3**.

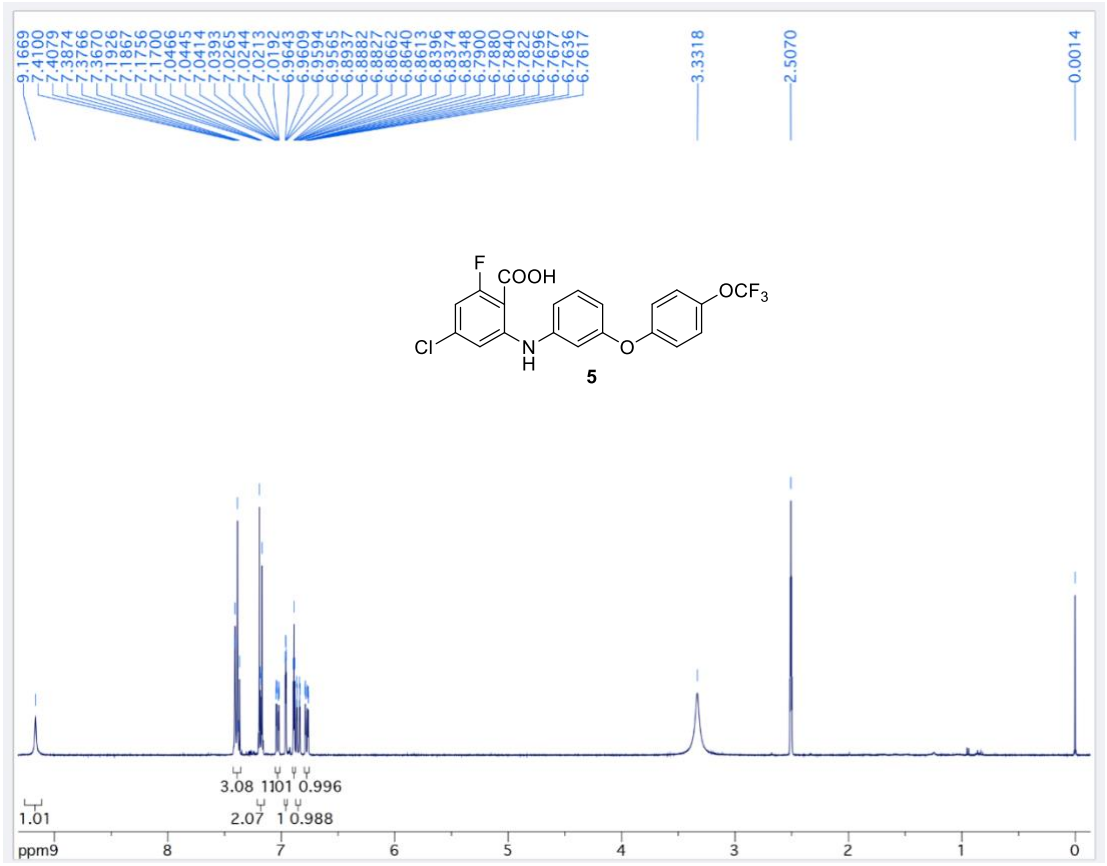


Figure S4. ^{1}H NMR spectrum of intermediate **5** (DMSO-*d*$_6$, 400 MHz).

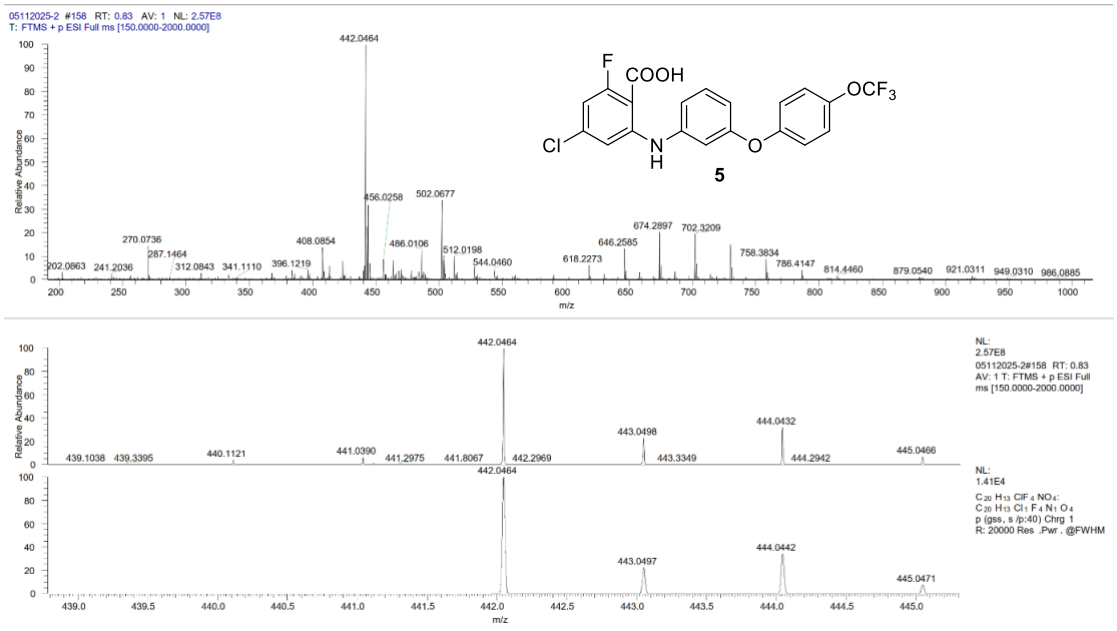


Figure S5. HRMS (ESI) of intermediate **5.**

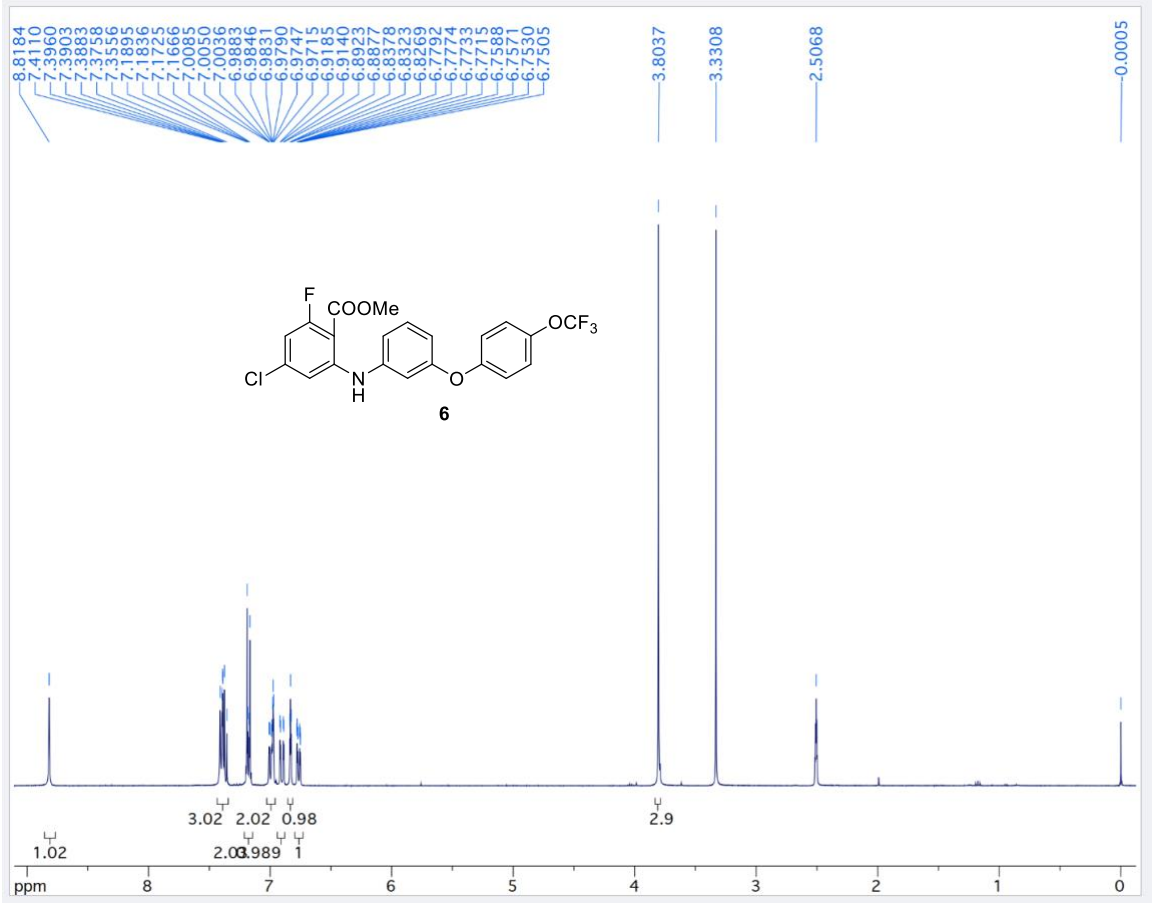


Figure S6. ^{1}H NMR spectrum of intermediate **6** (DMSO-*d*$_6$, 400 MHz).

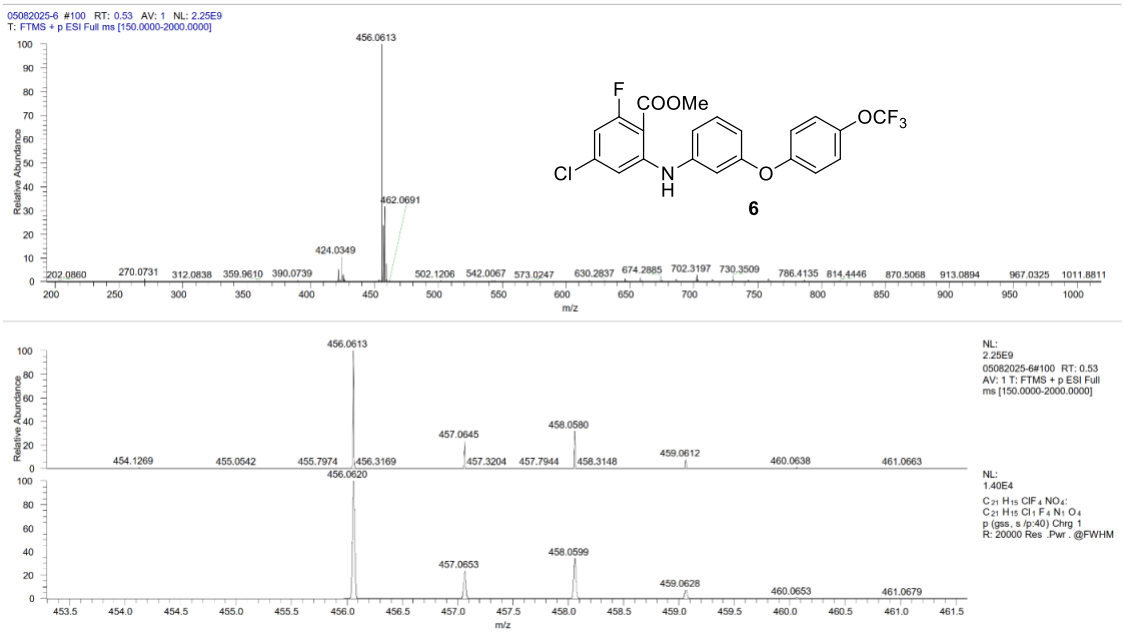


Figure S7. HRMS (ESI) of intermediate **6.**

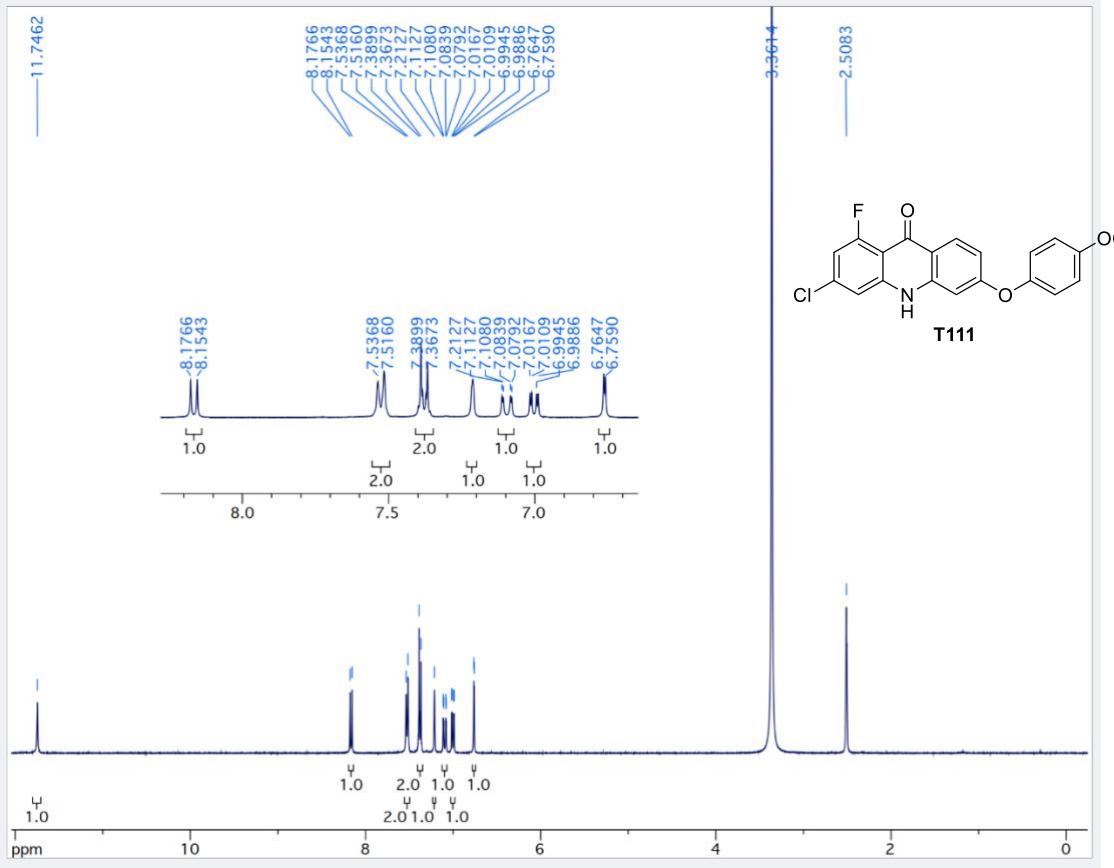


Figure S8. ^{1}H NMR spectrum of T111 (DMSO-*d*$_6$, 400 MHz).

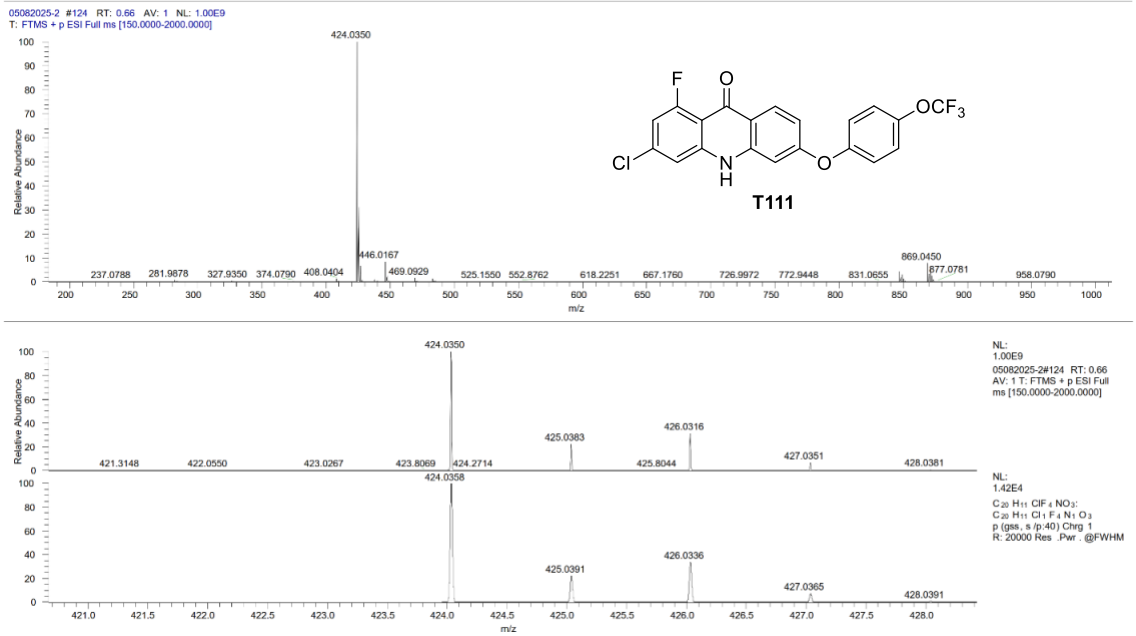


Figure S9. HRMS (ESI) of T111.

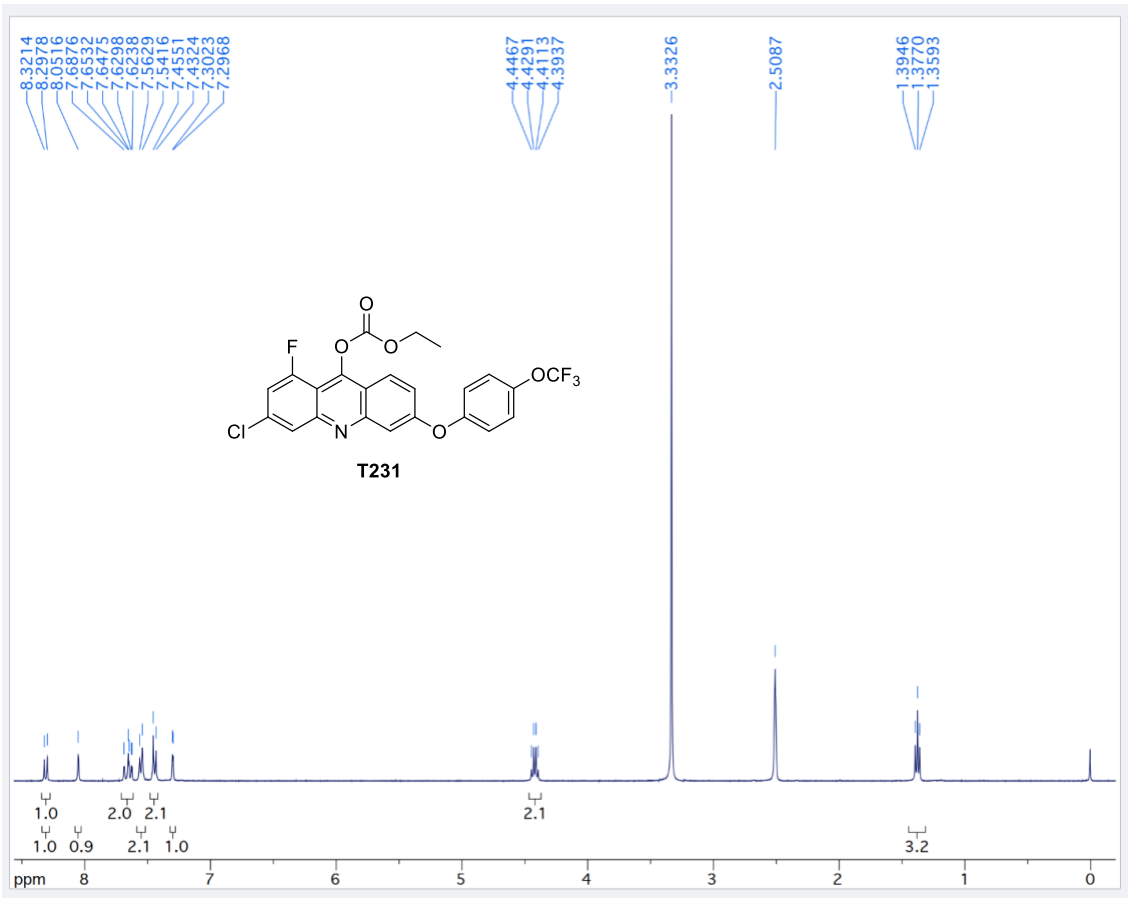


Figure S10. ^{1}H NMR spectrum of T231 (DMSO-*d*$_6$, 400 MHz).

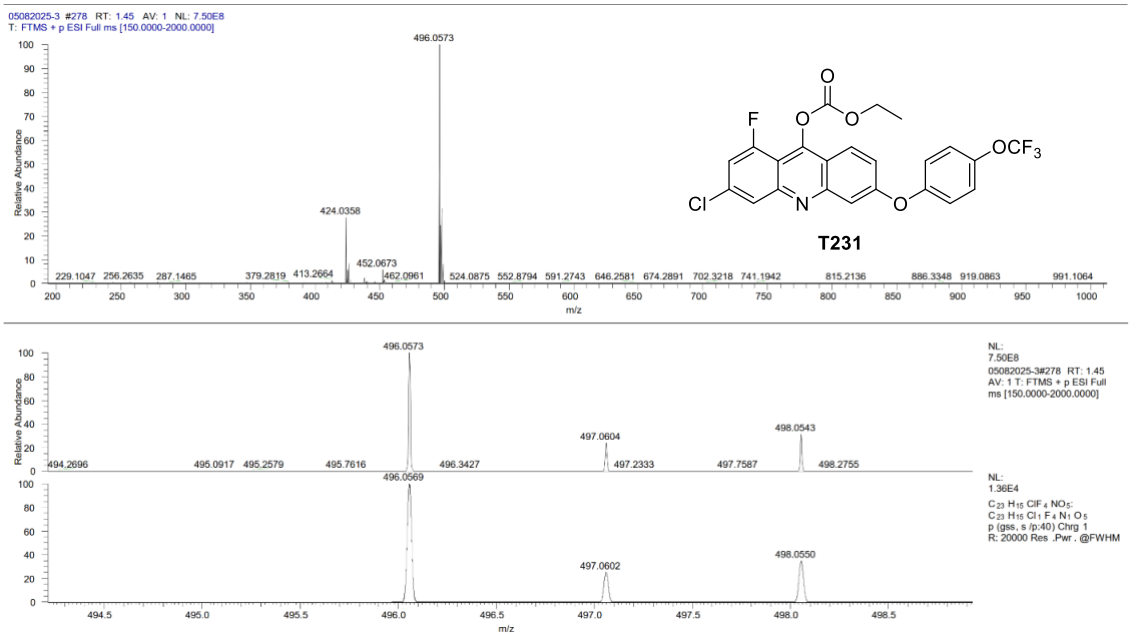
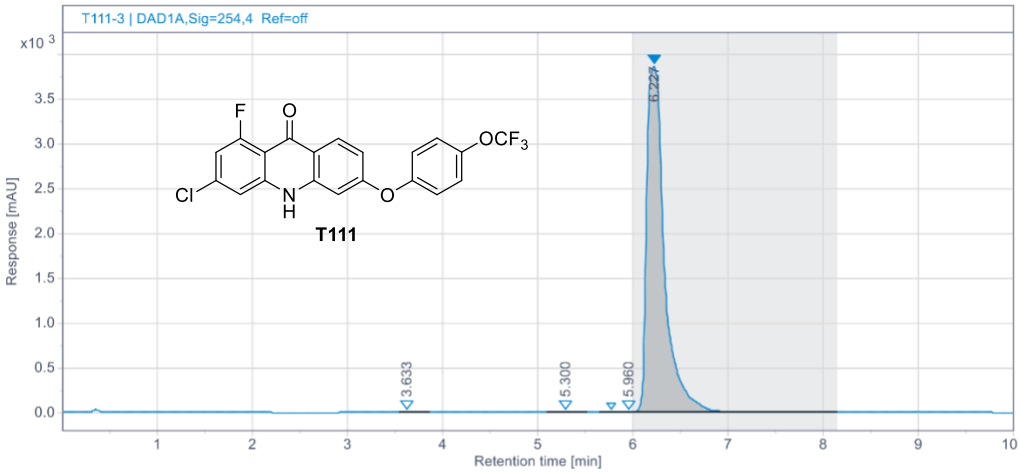
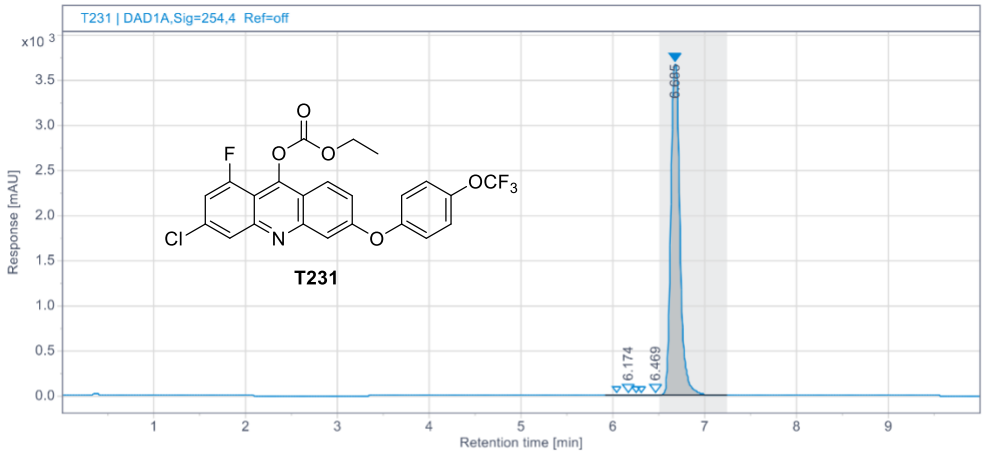


Figure S11. HRMS (ESI) of T231.



#	RT (min)	Area (mAU·s)	Area%	Height (mAU)	Height%	Start time (min)	End time (min)
5	6.227	49730.26	99.825	3857.079	99.65	6.011	8.157
4	5.96	22.516	0.045	3.995	0.1	5.85	6.011
3	5.78	14.541	0.029	2.677	0.07	5.653	5.85
2	5.3	30.578	0.061	4.954	0.13	5.1	5.523
1	3.633	19.736	0.04	1.814	0.05	3.554	3.877

Figure S12. HPLC traces of T111.



#	RT (min)	Area (mAU·s)	Area%	Height (mAU)	Height%	Start time (min)	End time (min)
6	6.685	25389.67	99.665	3671.369	99.47	6.517	7.25
5	6.469	23.769	0.093	5.519	0.15	6.381	6.517
4	6.312	7.363	0.029	1.96	0.05	6.28	6.381
3	6.255	5.682	0.022	1.987	0.05	6.23	6.28
2	6.174	23.786	0.093	5.176	0.14	6.116	6.23
1	6.052	24.792	0.097	4.851	0.13	5.926	6.116

Figure S13. HPLC traces of T231.