Supplemental Materials

Concentration-dependent early anti-vascular and anti-tumor effects of itraconazole in non-small cell lung cancer

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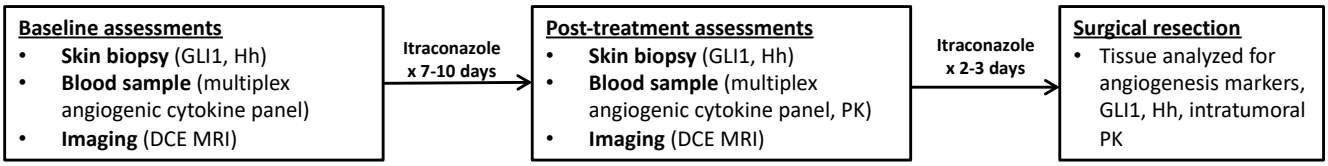
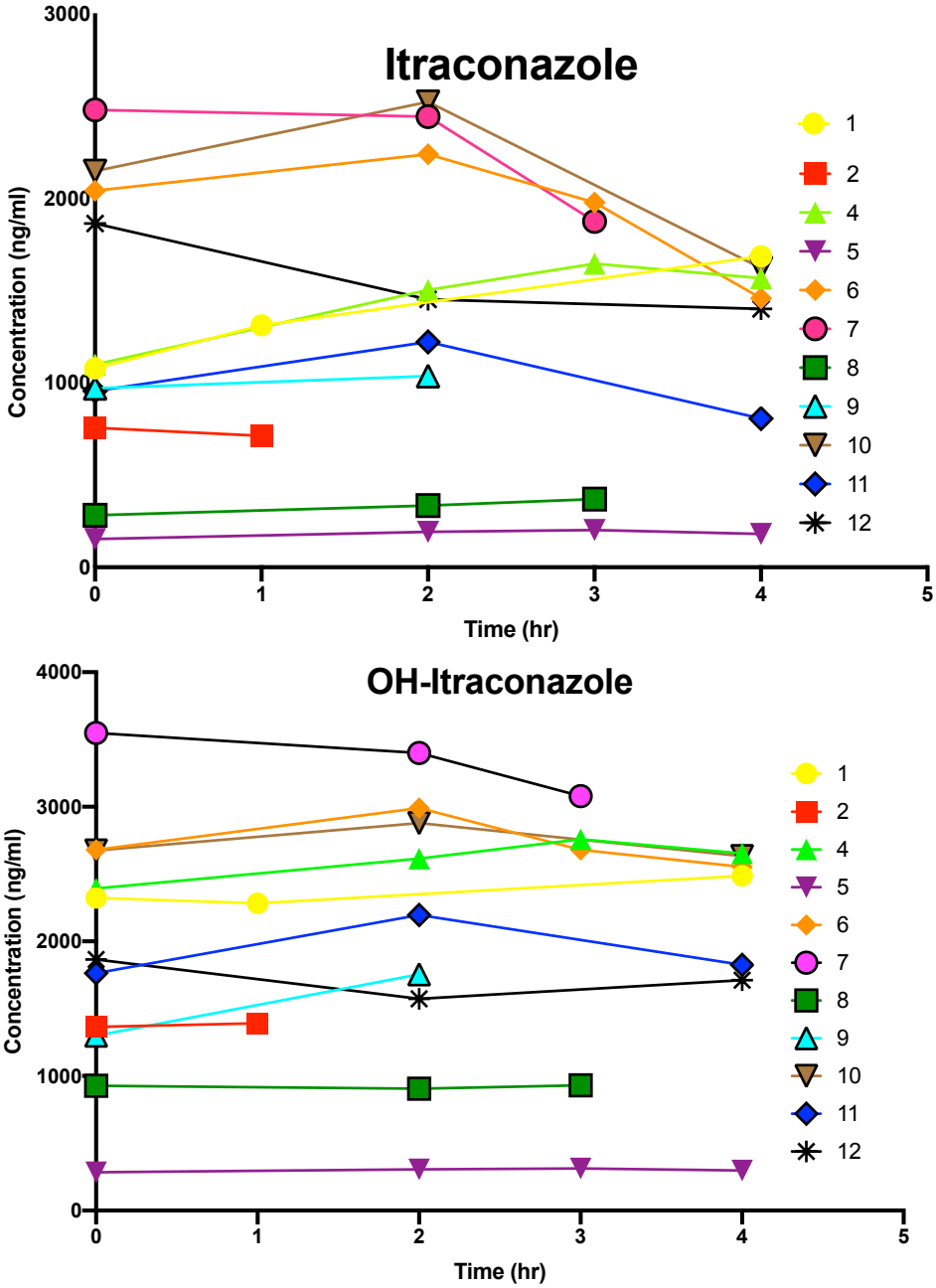
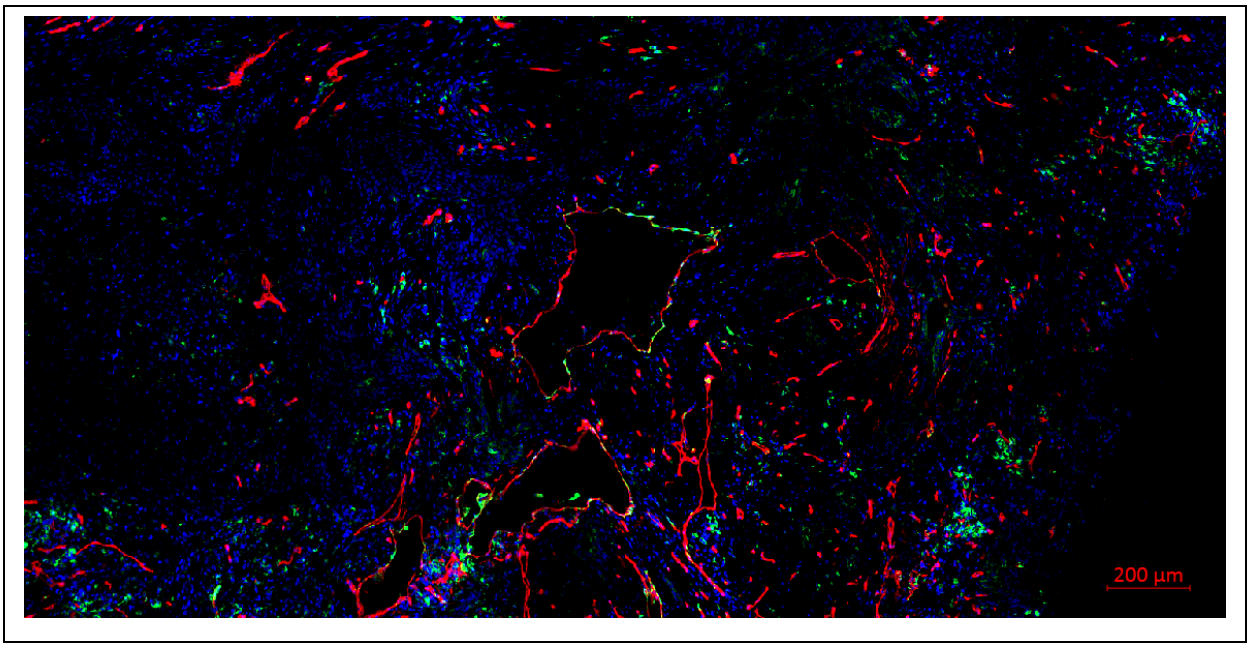
Supplemental Figures and Tables.



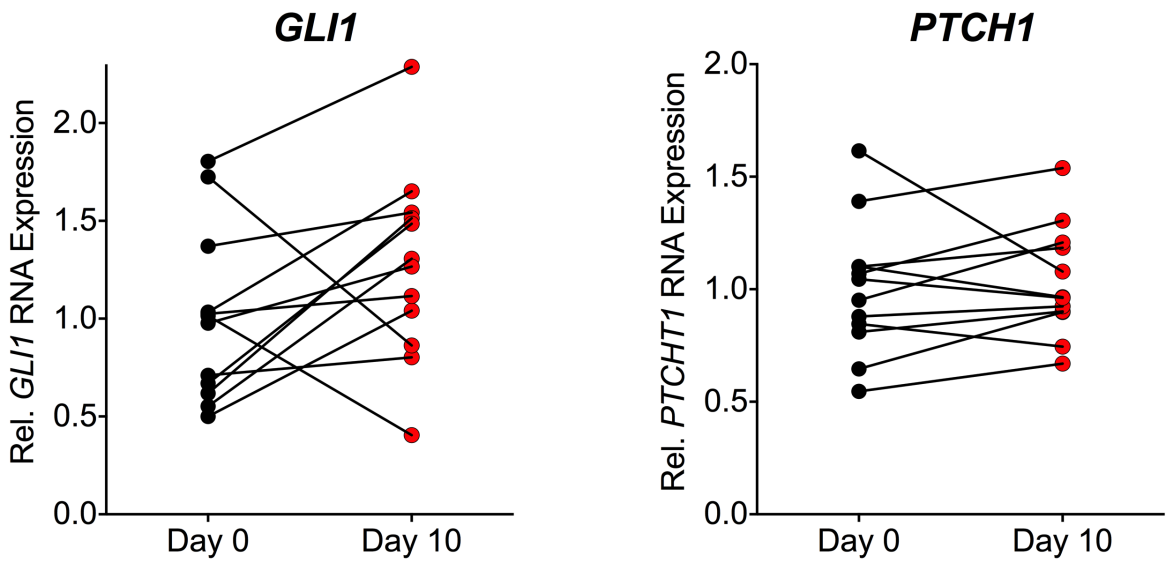
Supplemental Figure 1. Study schema.



<u>Supplemental Figure 2</u>. Representative chromatograms of itraconazole and hydroxy-itraconazole from plasma PK analysis.



<u>Supplemental Figure 3</u>. Representative post-itraconazole tumor specimen used to calculate mean vessel density. FFPE sections were evaluated for expression of CD31 (red) and CD34 (green) by immunohistochemistry. Vesselss showing co-expression of CD31 and CD34 were counted as positive.



Supplemental Figure 4. Changes in GLI1 and PTCH1 expression in paired skin biopsies in individual cases. *GLI1* and *PTCH1* mRNA transcription of skin biopsy samples were monitored on day 0 and day 10 of itraconazole treatment by qPCR. Connecting lines indicate samples from the same patient. All samples were normalized to the mean of respective day 0 mRNA levels.

Supplemental Tables

Supplemental Table 1. Pharmacokinetic parameters of itraconazole (**Supplemental Table 1a**) and hydroxyitraconazole (**Supplemental Table 1b**) at steady state. *n* refers to number of time-points.

1a. Itraconazole

Parameter	Subject										
	1	2	4	5	6	7	8	9	10	11	12
n	3	2	4	4	4	3	3	2	3	3	3
C_{min} (ng/mL)	1,077	712	1,098	153	1,457	1,873	282	969	1,620	806	1,399
C_{ave} (ng/mL)	1,357	734	1,453	182	1,927	2,263	328	1,003	2,095	994	1,570
C_{max} (ng/mL)	1,684	756	1,644	202	2,237	2,477	369	1,036	2,520	1,221	1,862
$AUC_{0\text{-}4h}$ (ng·h/mL)	5,687	2,936	5,778	733	8,099	9,432	1,292	4,012	8,805	4,202	6,161
Tissue (ng/g)	3,799	2,005	7,094	138	2,142	2,713	1,244	1,404	N/A	2,728	N/A

1b. Hyroxy-itraconazole

Parameter	Subject										
	1	2	4	5	6	7	8	9	10	11	12
n	3	2	4	4	4	3	3	2	3	3	3
C_{min} (ng/mL)	2,283	1,364	2,393	285	2,554	3,079	905	1,300	2,633	1,764	1,573
C_{ave} (ng/mL)	2,364	1,378	2,606	301	2,726	3,343	921	1,528	2,729	1,929	1,717
C_{max} (ng/mL)	2,486	1,392	2,759	313	2,990	3,549	931	1,756	2,879	2,196	1,865
$AUC_{0\text{-}4h}$ (ng·h/mL)	9,457	5,512	10,403	1,208	11,122	13,584	3,668	6,112	11,067	7,982	6,723
Tissue (ng/g)	4,338	1,828	7,002	124	2,420	2,853	1,782	1,837	N/A	3,528	N/A

Supplemental Table 2. Pre- and post-itraconazole MRI measurments

Patient	MR Measurements								
	Tumor max length (cm)			Volume (cm^3)			K^{trans} (min^{-1})		
	Pre	Post	% change	Pre	Post	% change	Pre	Post	% change
1	3.11	2.90	-6.8	13.44	13.92	+3.6	0.29	0.29	0
3	3.18	3.01	-5.3	13.83	11.92	-13.8	0.17	0.12	-29.4
4	4.26	4.41	+3.5	20.01	21.13	+5.6	0.22	0.16	-27.3
5	1.52	1.93	+27.1	3.14	3.54	+12.7	0.08	0.09	+12.5
6	2.64	2.45	-7.2	6.09	6.05	-0.7	0.11	0.10	-9.1
8	1.13	1.48	+31.0	2.49	2.24	-10.0	0.10	0.10	0
10	1.26	1.00	-20.6	1.13	0.84	-25.7	0.01	0.01	0
11	2.38	2.32	-2.5	8.07	7.29	-9.7	0.19	0.17	-10.5
12	2.74	2.67	-2.6	5.54	6.12	+10.5	0.32	0.51	+59.4

Supplemental Methods

PK methods

Chemicals and reagents

Optima LC-MS grade acetonitrile, water and methanol were purchased from Fisher Scientific (New Jersey, USA). Analytical grade formic acid was purchased from Acros Organics (New Jersey, USA). Itraconazole and hydroxyitraconazole were purchased from Selleck Chemicals and Toronto Research Chemicals, respectively. Posaconazole was obtained from Sigma. All other chemicals and reagents were of analytical grade and used without further purification. Blank human plasma and blank human lung tissue matrix was procured from Bioreclamation, Inc. (New York, USA).

Preparation of stock and working solutions of analytes and internal standard

Primary stock solutions of itraconazole and hydroxyitraconazole for the calibration curve (CC) and quality control samples (QC) were prepared by accurately weighing itraconazole or hydroxyitraconazole and dissolving in sufficient dimethylsulfoxide to yield a 200 µg/mL stock solution. Stock solutions of itraconazole and hydroxyitraconazole were stored at -20 °C, and subsequent dilutions were conducted using acetonitrile:water (70:30, v/v).

Primary stock solutions of the bioanalytical method's internal standard (IS) posaconazole were prepared by accurately weighing posaconazole and dissolving in chloroform to yield a 1000 µg/mL stock solution. Stock solutions of posaconazole were stored at -20 °C, and subsequent dilutions were conducted using acetonitrile:water (70:30, v/v).

For spiking of plasma samples with posaconazole, a working stock solution of 50 ng/mL posaconazole in toluene was prepared stored at room temperature. For spiking of tissue samples with

posaconazole, a working stock solution of 100 ng/mL posaconazole in DMSO was prepared stored at room temperature.

Preparation of calibration curve and quality control samples for analysis of plasma samples

Itraconazole and hydroxyitraconazole were validated over two calibration ranges to support low concentration and high concentration samples. Calibration curve I ranged from 3.9 to 2000 ng/mL and Calibration curve II raged from 125 to 4000 ng/mL. Calibration curve I samples were prepared by spiking 50µL of the working stock (40 µg/mL itraconazole/ hydroxyitraconazole in 70/30 acetonitrile:water) into 950 µL of plasma. This represented the top calibration curve point (i.e., the upper limit of quantification or ULOQ). The remaining calibration curve samples were prepared by serial dilution of the ULOQ standard in plasma. Quality control were prepared in a similar fashion by spiking 75 µL of the working stock (40 µg/mL itraconazole/ hydroxyitraconazole in 70/30 acetonitrile:water) into 1925 µL of plasma. This represented the high-quality control standard (HQC). The medium-quality control standard (MQC) and low-quality control standard (LQC) were prepared by serial dilution of the HQC standard in plasma. Calibration curve II samples were prepared by spiking 20µL of the working stock (100 µg/mL itraconazole/ hydroxyitraconazole in DMSO) into 480 µL of plasma. This represented the top calibration curve point (i.e., the upper limit of quantification or ULOQ). The remaining calibration curve samples were prepared by serial dilution of the ULOQ standard in plasma. Quality control were prepared in a similar fashion by spiking 50 µL of the working stock (100 µg/mL itraconazole/ hydroxyitraconazole in DMSO) into 1950 µL of plasma. This represented the high-quality control standard (HQC). The medium-quality control standard (MQC) and low-quality control standard (LQC) were prepared by serial dilution of the HQC standard in plasma. Spiking volume of the working standard did not exceed 5% of the matrix volume. QCs for Calibration curve I and II were prepared at 4, 12, 750 and 1500 ng/mL and 400, 1500 and 2500 ng/mL concentrations in plasma, respectively.

Preparation of calibration curve and quality control samples for analysis of tissue samples

The method for quantitation of itraconazole and hydroxyitraconazole was validated over the range 3.9 to 2000 ng/mL. Calibration curve samples were prepared by spiking 50 µL of the working stock (40 µg/mL itraconazole/ hydroxyitraconazole in 70/30 acetonitrile:water) into 950 µL of blank lung tissue (blank lung tissue was prepared in the same manner as clinical tissue samples). This represented the top calibration curve point (i.e., the upper limit of quantification or ULOQ). The remaining calibration curve samples were prepared by serial dilution of the ULOQ standard in blank tissue. Quality control were prepared in a similar fashion by spiking 75 µL of the working stock (40 µg/mL itraconazole/ hydroxyitraconazole in 70/30 acetonitrile:water) into 1925 µL of blank tissue. This represented the high-quality control standard (HQC). The medium-quality control standard (MQC) and low-quality control standard (LQC) were prepared by serial dilution of the HQC standard in plasma. Spiking volume of the working standard did not exceed 5% of the matrix volume. Quality control samples were prepared at 4, 12, 750 and 1500 ng/mL concentrations in tissue, respectively.

Plasma and Tissue Sample Collection

Patients received a 300 mg dose of itraconazole twice a day (600 mg/day) for 10-14 days. Subjects were instructed to take with or immediately after a meal. On day 5, 6, 7, or 8 plasma samples were obtained at approximately 0, 2, 3 and 4 hours following a 300 mg oral dose of itraconazole. A concurrent tissue sample was obtained. Plasma and tissue samples were analyzed for itraconazole and hydroxyitraconazole concentrations using a validated LC-MS/MS method.

Plasma Sample Preparation

Toluene (600 µL) containing (50 ng/mL) posaconazole was added to a 50 µL aliquot of plasma. The resultant mixture was vortexed for 20 min at 2500 rpm, followed by centrifugation at 5000 rpm for 5 min. Five hundred microliters (500µL) of the clear supernatant, was removed, placed in a new

Eppendorf tube and dried using a SpeedVac. Samples were reconstituted using 200 µL of acetonitrile:water (70:30).

Tissue Sample Preparation

Frozen lung tissue (blank or study sample) was placed on a clean and dry glass plate. The tissue was cut into small sections of approximately 50 mg and then accurately weighed. The sample was placed into a 1.5 mL Eppendorf centrifuge tube, add four volumes of water (200 µL) and homogenize the tissue using DPS-20 dual processing system from PRO Scientific INC., (Oxford, CT, USA).

To 50 µL of the tissue homogenate 20 µL of 2M ammonium acetate buffer was added and briefly vortexed. MTBE (600 µL) containing (50 ng/mL) posaconazole was then added to the mixture. The resultant suspension was vortexed for 30 min at 2500 rpm, followed by centrifugation at 5000 rpm for 5 min. Five hundred microliters (500µL) of the clear supernatant, was removed, placed in a new Eppendorf tube and dried using a SpeedVac. Samples were reconstituted using 200 µL of acetonitrile:water (70:30).

HPLC operating conditions

A Shimadzu CBM-20A Nexera X2 series LC system (Shimadzu Corporation, Kyoto, Japan) equipped with degasser (DGU-20A) and binary pump (LC-30AD) along with auto-sampler (SIL-30AC) and (CTO-30A) column oven. The autosampler was maintained at 10 °C. An injection volume of 1 µL was used and chromatographic separation was achieved using a Kinetex C18 (1.7 µm, 2.1 × 50mm) column. The mobile phase, consisting of 0.1% formic acid in water (pump A) and acetonitrile with 0.1% formic acid (pump B) used for the method. The mobile phase pumped using a gradient program at a flow rate of 0.5 mL/min into the mass spectrometer electrospray ionization chamber in positive polarity.

Gradient program initiated with 10% of B and maintained for 0.5min, then ramped to 90%B by 1.5min and maintained at 90%B until 2.5min, changed back to 10%B by 2.6min and maintained until 3min before getting back to initial gradient.

Mass spectrometry operating conditions

Quantitation was achieved employing electrospray ionization in positive ion mode for the analytes using a SCIEX QTRAP 6500^{+} mass spectrometer (Redwood, CA, USA) equipped with the Turbo V source operated at 550 ºC. The nebulizer gas, auxiliary gas, curtain gas, CAD gas were set at 50, 50, 40 psi and "medium", respectively. The declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were 100, 50, 10, 20 V for (itraconazole); 100, 52, 10, 20 V for (Itraconazole-1); 100, 50, 10, 26 V for (Hydroxy itraconazole); 100, 51, 10, 15 V for (Hydroxy Itraconazole-1); and 100, 50, 10, 13 V for (posaconazole), respectively. Detection of the ions was carried out in the multiple-reaction monitoring mode (MRM), by monitoring the precursor > product transitions of: 705.4 > 392.4 and 705.4 > 256.1 (sum over 2 MRMs) for itraconazole; 721.3 > 408.3 and 721.3 > 683.3 (sum over 2 MRMs) for hydroxyitraconazole, and 701.204 > 683.3 for posaconazole. The data obtained were processed by Analyst software™ (version 1.6.3).

Method validation

The methods for analysis of itraconazole and hydroxyitraconazole in plasma and tissue were validated according to the United States FDA's May 2018 Guidance for Industry on "Bioanalytical Method Validation." The parameters of sensitivity, selectivity, matrix effect, linearity, accuracy, precision, recovery, dilution integrity and stability were studied and all parameters met the acceptance criteria set forth in the FDA's Guidance for Industry.

Pharmacokinetic analysis

The pharmacokinetic parameters of itraconazole and hydroxyitraconazole in plasma were estimated using the Phoenix 64 WinNonLin 6.4 (Certara USA, Clayton, MO, USA) software package. The steady state concentration vs. time results from the bioanalysis were fit to a non-compartmental model. The determined pharmacokinetic parameters for plasma included: (1) area under the curve calculated from 0 to 4 hours ($AUC_{0\text{-}4h}$); (2) maximum plasma drug concentration (C_{max}); (3) average plasma drug concentration (C_{ave}), and (4) minimum plasma drug concentration (C_{min}).