Electronic Supporting Information

Sulfasalazine in Ionic Liquid Form with Improved Solubility and Exposure

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Materials and Methods

Sulfasalazine, choline chloride, choline hydroxide (45% in methanol), β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), sodium chloride, magnesium chloride, hydrochloric acid, dimethylsulfoxide, acetonitrile, formic acid, sodium hydroxide, (hydroxypropyl)-methyl cellulose, and tween were purchased from Sigma Aldrich (St. Louis, MO, USA). Stable isotope of d4-Sulfasalazine was purchased from Toronto Research Labs (Toronto, Canada). N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid and Lucifer Yellow were purchased from Life Technologies, (Carlsbad, CA, USA). Liver S9 fractions from Sprague-Dawley (SD) rats were purchased from XenoTech (Lenexa, KS, USA). The Caco-2 cells (Passage No. 18) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Plasma samples for standard curves or for dilution purposes were purchased from Bioreclamation (Hicksville, NY, USA).

Nuclear Magnetic Resonance Spectroscopy (NMR)

^{1}H spectrum was collected utilizing a Bruker (Madison, WI, USA) spectrometer 500 MHz Bruker Avance Spectrometer Bruker/Magnex UltraShield 500 MHz magnet operating at 500 MHz.

Thermogravimetric Analysis (TGA)

TGA analyses were performed on a Mettler-Toledo (Columbus, OH, USA) TGA/DSC 1. The instrument's internal temperature was calibrated by observing the melting point of Au, Zn, and In (melting points 1064 °C, 419.5 °C, and 155.6 °C, respectively) standards. The samples were analyzed in 70 μL alumina pans using nitrogen as a purge gas. The sample (2.73 mg) was heated from room temperature to 800 °C and measured in the

dynamic heating regime, using a constant heating ramp of 5 °C min^{-1} with a 30 min isotherm at 75 °C. Decomposition temperatures are reported as onset to 5 wt% mass loss ($T_{onset\ 5\%}$).

Differential Scanning Calorimetry (DSC)

Thermal transitions were measured on a Mettler Toledo DSC1 Star unit under a stream of nitrogen. The sample (3.74 mg) was placed in closed aluminum pan perforated with a pinhole. A typical cycle consisted of initially cooling the samples from 25 to -50 °C at a cooling rate of 5 °C/min, a 5 min isotherm, heating at a rate of 5 °C/min to 125 °C, and a final 5 min isotherm at 125 °C. The cycle was repeated three times to ensure consistency of the observed thermal transitions.

X-Ray Power Diffraction

X-ray powder diffraction (XRPD) patterns were collected using a Bruker AXS (Madison, WI, USA) D8 Advance X-ray Diffractometer equipped with LynxEye detector and copper K-alpha (Cu Kα) radiation at 40 kV and 40 mA. A powder sample was gently flattened at the center of a sample holder for diffraction measurement. The sample was run as a continuous scan from 2.9° to 29.6° 2θ angle with step size of 0.025° 2θ and data collection time of 0.4 seconds per step. All data analysis was performed using DIFFRAC.EVA (version 2.1) software (Bruker AXS).

Experimental details

Sulfa-IL Synthesis and Characterization

Sulfasalazine (0.25 g, 0.63 mmol) was suspended in 15 mL of methanol and a 45% solution of choline hydroxide in methanol (0.63 mmol) was added dropwise. The suspension was stirred for 2 h at room temperature until a clear red solution was obtained. The remaining volatile material was removed under reduced pressure (0.01 mbar, 40 °C) to yield quantitatively choline sulfasalazine as red glassy solid.

^{1}H NMR (500 MHz, DMSO-d_6) δ = 8.27 (d, *J* = 2.7 Hz, 1H), 7.99 (d, *J* = 8.6 Hz, 3H), 7.87 (d, *J* = 8.6 Hz, 2H), 7.80 (dd, *J* = 8.8, 2.7 Hz, 1H), 7.77 – 7.71 (m, 1H), 7.21 (d, *J* = 8.7 Hz, 1H), 6.86 (t, *J* = 6.2 Hz, 1H), 6.73 (d, *J* = 8.8 Hz, 1H), 5.35 (br, 1H), 3.84 (m, 2H), 3.44 – 3.37 (m, 2H), 3.33 (s, 2H), 3.11 (s, 9H). $T_{onset\ 5\%}$ = 214 °C; T_g = 59 °C

Solubility Testing

Sulfasalazine powder (in triplicates ~5 mg) was added to 1 mL of carrier (saline, phosphate buffer, polyethylene glycol, or sulfobutyl ether beta cyclodextrin), or Sulfa-IL (in triplicates ~ 60 mg) was added to 1 mL of saline solution. The solution mixtures were shaken for 1 h at room temperature, then centrifuged at 13000 rpm for 10 min, and the supernatant was diluted 10-1000 times depending on the concentration, and analyzed on LC/MS using Method A (see the Bioanalytical Procedures section).

Metabolic Stability Study Using Rat Liver S9 Fractions

The metabolic stability study was carried out using SD rat liver S9 fractions. Sulfa, Sulfa-PM, or Sulfa-IL in DMSO solutions were added to a mixture of 0.1 M phosphate buffer at pH 7.4 and 37 °C, NADPH (2 mM), and $MgCl_2$ (3 mM). The final concentration of sulfasalazine in each incubation was 1 μM. The reactions were initiated by adding liver S9 (2.5 mg/mL, final). Reactions were terminated at 0, 5, 10, 20, 30, 45, and 60 min by adding an equal volume of acetonitrile (ACN) containing 1 mM carbutamide (internal standard [IS]).

The samples were then refrigerated at approximately 4 °C for 30 min and then centrifuged at 1800 g for 10 min keeping the temperature constant (4 °C). The peak area of Sulfa in the supernatants was measured by liquid chromatography with tandem mass spectrometry (LC/MS). The half-life ($t_{1/2}$) for the disappearance of Sulfa was calculated from the plot of the natural logarithmic-transformed concentration of Sulfa versus the incubation time. LC/MS analysis was carried out using Method B (see the Bioanalytical Procedures section).

Metabolic stability was expressed as in vitro half-life ($t_{1/2}$) and was determined from the disappearance of Sulfa during incubation with S9 fractions. The peak areas of Sulfa in aliquots terminated at 0 min were defined as 100%, and the percentage of the parent compound remaining at other times was calculated by comparing peak areas at these times with those at 0 min. The rate constant (k) for the disappearance of Sulfa was estimated from the plot of the percentage of Sulfa remaining at each time and the $t_{1/2}$ was calculated using eq. 1:

$$t_{1/2} = (\ln 2)/k \quad (1)$$

The slope of the lines from time vs. concentration plots indicate that sulfasalazine in the three formulations underwent the same extent of metabolism and had similar half lives (Fig. 1, main manuscript). The calculated half lives for Sulfa, Sulfa-PM, and Sulfa-IL are 36.4 min, 36.6 min, and 36.9 min, respectively.

Caco-2 cells permeability test

The Caco-2 cells were prepared according to internal protocols. Briefly, bidirectional transport studies were performed at 37 °C in an atmosphere of 5% CO_2 in air and 90% humidity. Before each experiment, the confluent cell monolayers on Transwell inserts were washed and equilibrated for 60 min with transport media (Hank's balanced salt solution [HBSS] containing 10 mM of N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid [HEPES] and 10 mM glucose, pH 7.4.

The experiment was initiated by adding a solution containing the test compounds (Sulfa or Sulfa-IL at 50 μM with 50 μM of Lucifer Yellow (LY)) to either the apical (for A-to-B transport) or basolateral (for B-to-A transport) compartment. LY was used as a paracellular transport control to monitor cell integrity. At 30 and 60 min, the sample aliquots of receiving solutions were withdrawn from the basolateral side (for A-to-B transport) or from the apical side (for B-to-A transport), and replaced immediately with an equal amount of fresh transport media except at the 60-min time point (the end of the incubation). All experiments were performed in triplicate.

Sulfa alone underwent low A-to-B ratio of 0.23×10^{-6} cm/sec (SEM 0.01) and higher B-to-A ratio of 9.15×10^{-6} cm/sec (SEM 0.03). Sulfa-IL showed very similar permeability pattern: low A-to-B ratio of 0.24×10^{-6} cm/sec (SEM 0.91) and B-to-A ratio of 10.65×10^{-6} cm/sec (SEM 0.84).

Pharmacokinetics Study

All animal procedures were approved by the Millennium Institutional Animal Care and Use Committee (IACUC). Male Sprague-Dawley (SD) rats (250–350 g) were purchased from Charles River (Wilmington, MA, USA). Femoral artery and femoral vein cannulations were performed for blood sampling and intravenous dosing, respectively. Furthermore, in biliary excretion studies, bile ducts were also cannulated. Surgeries on rats were performed by the Comparative Medicine group at Millennium Pharmaceutics. Animals in each study were matched based on age and body weight.

All *in vivo* doses are expressed as mg of sulfasalazine/kg after correction for the molecular weight of the salt form. Sulfa-PM or Sulfa-IL were given via the I.V. route to rats. Due to

limited solubility, Sulfa-PM was formulated in 20% SBE cyclodextrin and was dosed at 0.5 mg/kg. For comparison, Sulfa-IL was also dosed at the same dose in saline. Both dosing groups exhibited similar exposures at this equivalent dose. The higher solubility of Sulfa-IL allowed higher doses of 6.0, 12.0, and 24.0 mg/kg and AUC increased linearly with dose (Fig. 2, main manuscript). The PK parameters (calculated using Phoenix software, 6.3.0.395) are shown in Table S1.

Table S1. PK parameters obtained after dosing SD rats with 0.5 mg/kg of Sulfa-PM or incremental doses of Sulfa-IL. All doses were adminstered via the I.V. route.

	Sulfa-PM 0.5 mg/kg	Sulfa-IL 0.5 mg/kg	Sulfa-IL 6.0 mg/kg	Sulfa-IL 12.0 mg/kg	Sulfa-IL 24.0 mg/kg
Cl (L/h/kg)	0.55 ±0.15	0.78±0.15	0.69±0.07	0.68±0.04	0.87±0.07
Vss (L/kg)	0.20±0.02	0.17±0.03	0.29±0.10	0.28±0.05	0.38±0.07
$t_{1/2}$ (h)	0.99±0.17	0.50±0.20	1.95±0.99	2.89±1.96	6.33±3.47
AUC_{inf} (h*nM)	2440±650	1660±340	21800±2060	44200±2380	69200±5180

Cl = the clearance; Vss = the volume of distribution at a steady state; $t_{1/2}$ = the half life; AUC = the area under curve of sulfasalazine concentration extrapolated to infinity.

To determine if the increased solubility actually resulted in better absorption that was obscured by increased biliary clearance, bile-duct cannulated rats were treated with 50 mg/kg Sulfa-PM and Sulfa-IL separately. Sulfasalazine excretion in bile was about 1.5% and 1.8% of the dose for Sulfa-PM and Sulfa-IL, respectively.

Drug Administration and Plasma Collection

The plasma concentration time course was determined over 48 h in an intravenous/oral crossover study. Randomly assigned rats were treated via I.V. bolus injection with: a) Sulfa (0.5 mg/kg; 1 mL/kg of 20% sulfobutyl ether cyclodextrin (SBE), and 0.1% DMSO) co-administered with choline hydroxide (0.33 mg/kg), or b) Sulfa–IL (0.5, 6.0, 12.0, or 24.0 mg/kg at 1 mL/kg in saline). The jugular vein samples were collected at the following times after dose administration: predose, 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 24, and 48 h, and sulfasalazine concentration was measured in plasma using Method C (see the Bioanalytical Procedures section).

Oral administration of Sulfa-IL and Sulfa-PM to intact rats or bile duct cannulated rats was given via gavage. Sulfa-PM suspension was prepared at 10 mg/mL by mixing 50 mg/kg of sulfasalazine with 0.5% (hydroxypropyl)-methyl cellulose, 0.2% tween, and choline hydroxide (33 mg/kg). The Sulfa-IL solution was prepared at 10 mg/mL by dissolving 63 mg/kg of Sulfa-IL in saline, the active equivalent of 50 mg/kg of Sulfa-PM. The jugular vein samples were collected at the following times after dose administration: 0.25, 0.5, 1, 2, 4, 6, 8, 24, and 48 h, and the resulting plasma was analyzed for sulfasalazine. All samples were analyzed using Method C, under the bioanalytical procedures.

Dissolution of Sulfasalazine in Simulated Gastrointestinal Fluids

Sulfa samples in water were prepared at 30 mg/mL equivalent of sulfasalazine using commercially available sulfasalazine, Sulfa-PM, or Sulfa-IL. To determine the dissolution in simulated gastric fluid (SGF), sulfasalazine samples in water were mixed with SGF (1:1 ratio) and stirred for 60 min at 37 °C. Sample aliquots were taken at 15, 30, and 60 min and centrifuged at 13000 rpm for 10 min. The supernatants were diluted for LC/MS analysis to determine the concentrations of dissolved sulfasalazine (see Method B, the Bioanalytical Procedures section). A greater concentration of dissolved sulfasalazine in water was obtained

for Sulfa-IL (1.34 mg/mL) than that for sulfasalazine or Sulfa-PM (0.008 mg/mL) (Fig. 3, left, main manuscript). The pH values of samples measured at room temperature after 60 min, were 1.5, 1.5, and 6.6 for Sulfa, Sulfa-PM, and Sulfa-IL, respectively. To determine the dissolution of sulfasalazine in SGF and simulated intestinal fluid (SIF) mixture, sulfasalazine samples in water were first mixed with SGF at a 1:1 ratio, as described above, and stirred for 60 min at 37 °C. Then SIF was added to make a 1:3 ratio of SGF:SIF and samples were stirred for 3 h at 37 °C. Sample aliquots were taken at 0.5, 1, 2, and 3 h after addition of SIF, and centrifuged at 13000 rpm for 10 min. The supernatants were diluted for LC/MS analysis to determine the concentrations of dissolved sulfazalazine. The concentrations of dissolved sulfasalazine from Sulfa and Sulfa-PM samples were 0.86 mg/mL and the concentration of sulfasalazine dissolved from Sulfa-IL was 3.63 mg/mL (Fig. 3, right, main manuscript). The pH values of the samples after mixing with SIF were 6.1, 6.1, and 6.5 for Sulfa, Sulfa-PM and Sulfa-IL, respectively.

Bioanalytical Procedures (developed to only detect the sulfasalazine ion in the sample)

Method A. Diluted samples were introduced to a Synergi Hydro-RP (75 x 4.6 mm, 4 µm, 80 Å; Phenomenex, Torrance, CA, USA) HPLC column with a CTC PAL autosampler (Leap Technologies; Carrboro, NC, USA) and an integrated HPLC pumping system (Agilent Technologie; Wilmington, DE, USA). Sulfasalazine was then eluted and detected by an API 4000 Q-trap mass spectrometer (Applied Biosystems/MDS Sciex; Foster City, CA, USA) fitted with a TurboIonSpray interface. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B), and the flow rate was 1 mL/min. The starting condition for the HPLC gradient was 85:15 (A/B), and this was held for 0.5 min. From 0.5 to 3.5 min, the mobile phase composition changed linearly to 15:85 (A/B). This condition was held until 3.6 min. The gradient was returned in a linear fashion to 85:15 (A/B) from 3.6 to 3.85 min and re-equilibrated until 5 min. The injection volume was 20 µL. Multiple reaction monitoring (MRM) was used to monitor Sulfa.

Method B. The amount of sulfasalazine in samples was quantified by liquid chromatography with tandem mass spectrometry (LC/MS/MS). The liquid chromatography with tandem mass spectrometry (LC/MS/MS) system consisted of an MDS SCIEX Triple Quad™ API 4500 mass spectrometer (Applied Biosystems, Concord, Ontario, Canada) coupled with an integrated HPLC pumping system (Agilent Technologie; Wilmington, DE, USA). Sulfasalazine was then eluted and detected by an API 4000 Q-trap mass spectrometer (Applied Biosystems/MDS Sciex; Foster City, CA, USA) fitted with a TurboIonSpray interface. Samples were analyzed using a Synergi Hydro-RP ((75 x 4.6 mm, 4 µm, 80 Å Phenomenex; Phenomenex, Torrance, CA, USA). The mobile phase, gradient, injection volume, and the reaction monitoring conditions are the same as in Method A.

Method C was used to analyze sulfasalazine from the PK samples and Caco-2 samples. The extracted samples were introduced to a Synergi Hydro-RP (75 x 4.6 mm, 4 µm, 80 Å; Phenomenex, Torrance, CA, USA) HPLC column with a CTC PAL autosampler (Leap Technologies; Carrboro, NC, USA) and an integrated HPLC pumping system (Agilent Technologie; Wilmington, DE, USA). Analytes were then eluted and detected by an API 4000-triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex; Foster City, CA, USA) fitted with a TurboIonSpray interface. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B), and the flow rate was 1 mL/min. The starting condition for the HPLC gradient was 95: 5 (A/B), and this was held for 0.5 min. From 0.5 to 3.5 min, the mobile phase composition changed linearly to 5:95 (A/B). This condition was held until 3.6 min. The gradient was returned in a linear fashion to 95: 5 (A/B) from 3.6 to 3.85 min and re-equilibrated until 5 min. The injection volume was 20 µL. Multiple reaction monitoring (MRM) was used to monitor Sulfa.

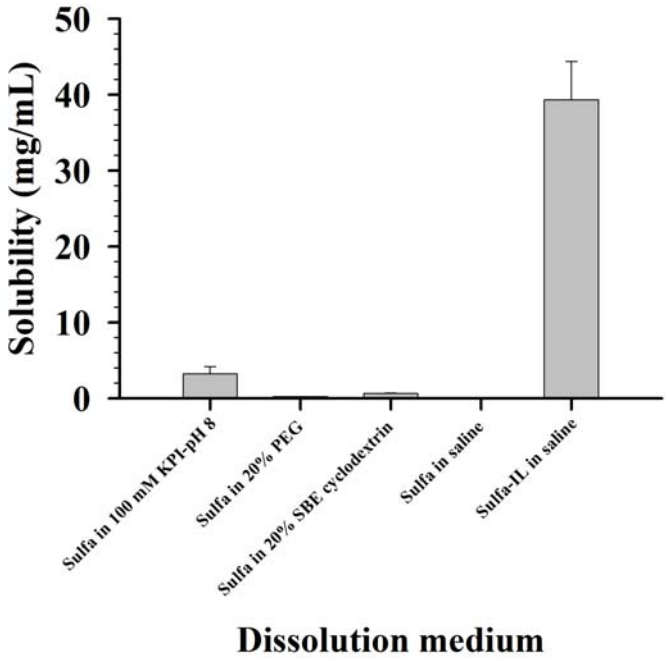

Fig. S1 Solubility of sulfasalazine (mg/mL) in various media. After mixing for one hour, the samples were centrifuged and sulfasalazine concentration in the supernatant was measured using LC/MS.