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Supporting Information

Elasticity in Macrophage-Synthesized Biocrystals

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ange_201611195_sm_miscellaneous_information.pdf
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ange_201611195_sm_Movie_2.wmv

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References mentioned in the supplementary document have the same index as in the main manuscript document.

Materials and Methods

Mouse Model for Generating Biocrystals. Macrophages containing biocrystals were generated as published before[23,25,37] Mice (4 week old, male C57Bl/6) were purchased from the Jackson Laboratory (Bar Harbor, ME) and acclimatized for 1 week in a specific-pathogen-free animal facility. Animal care was provided by the University of Michigan's Unit for Laboratory Animal Medicine (ULAM). The experimental protocol was approved by the Committee on Use and Care of Animals and all procedures were carried out in accordance with the approved protocol. Clofazimine (CFZ) (C8895; Sigma-Aldrich, St. Louis, MO) was dissolved in sesame oil (Roland, China, or Shirakiku, Japan) to achieve a concentration of 3 mg/ml, which was mixed with Powdered Lab Diet 5001 (PMI International, Inc., St. Louis, MO) to produce a 0.03% drug to powdered chow feed.

Peritoneal Lavage to obtain Peritoneal Macrophages. Peritoneal lavage was done 8 weeks after the initiation of CFZ treatment. Mice were euthanized by exsanguination while deeply anesthetized by an intraperitoneal injection of ketamine (100 mg/kg)/xylazine (10 mg/kg) followed by sterilization of the outer skin with 70% of ethanol. A small incision was made along the midline of the abdomen followed by abdominal skin retraction up to the thoracic boundary and the animal extremities to expose the intact peritoneal wall. A smaller incision was then made on the peritoneal wall to expose the cavity. The entire peritoneal cavity was washed with ice-cold sterile Phosphate Buffered Saline (PBS) + 5% of Fetal Bovine Serum (FBS) (5–10 ml) and collected as peritoneal exudate. The exudate was then centrifuged (100 x *g* for 5 min, 4 °C) and resuspended in 1.5 ml of PBS + 5% of FBS. Cells were counted using a hemocytometer for viable cells using Trypan Blue and for biocrystal-containing cells. For preparation of microscopy

slides, a 20 µl drop of cell suspension was placed on a glass slide and allowed to dry overnight in the dark. The following day, a single drop of Prolong® Gold (Life Technologies, Carlsbad, CA) was added to the dry slide and a cover slip was applied prior to imaging.

Alveolar Lavage to obtain Alveolar Macrophages. Mice were euthanized as described above and the trachea was surgically exposed and cannulated with an 18G luer stub and the lungs were lavaged to obtain alveolar exudate by instilling PBS containing 0.5 mM of EDTA in 1 ml aliquots for a total of 6 ml. The alveolar exudate fluid was centrifuged (400 x *g*, 10 min, 4 °C) and resuspended in RPMI 1640 media. Viable (using Trypan Blue staining method) and biocrystal containing cells were counted using a hemocytometer followed by preparation of microscope slides as mentioned above.

Isolation of Biocrystals. At 8 weeks post-drug feeding, mice were euthanized as described above and spleens were harvested and cut open to prepare tissue homogenate in phosphate-buffered saline (PBS). The tissue homogenate was sonicated for 30 min and centrifuged (100 x *g* for 1 min) to remove large cell debris. A solution of 10% sucrose in PBS was added to the acquired supernatant and the mixture was centrifuged (100 x *g*). The resulting supernatant was centrifuged (3200 x *g* for 20 min) to pellet drug inclusions which were then resuspended in 2 ml of 10% sucrose in water (w/v). CLDIs were further purified using a three-layer discontinuous gradient (50, 30, and 10% sucrose (w/v) in PBS) centrifugation method (3200 x *g* for 30 min, no brakes)[37].

Brightfield and Polarization Microscopy. The combination brightfield and diattenuation LC-Pol-Scope microscope set-up is a custom built microscopic imaging system similar to the birefringence LC-PolScope designed by Oldenbourg et al[34], but without the polarization analyzer. Our LC-PolScope is built on the Nikon Eclipse T*i* inverted microscope (Nikon Instruments, Melville, New York), with the computer-controlled universal compensator (Hinds Instrumentation, Hillsboro, Oregon) placed between the interference filter (623±22 nm, Semrock Optics, Rochester, New York) and condenser lens. Illuminating light is narrowed to 623 nm by the interference filter, and the light is linearly polarized by passing it through a universal compensator, allowing for the diattenuation of the sample to be measured. The LC in the universal compensator is controlled by Image J "Micro-manager" software (Vale Laboratory, UCSF) and is automatically rotated to produce polarized light at 0°, 45°, 90° and 135° angles, respective to the horizontal, during image acquisition. The image maps of diattenuation, mean transmittance, and angle of high transmittance are generated by image analysis algorithms followed by calibration and have been published before[32,34]. Brightfield and fluorescence images were captured using the Nikon DS-U3 camera (Nikon Instruments) and Photometrics CoolSnap MYO camera system (Photometrics, Tucson, Arizona), respectively, under the control of Nikon NIS-Elements AR software (Nikon Instruments). Microscopy slides of samples were prepared as mentioned before.

Measuring Curvature of Crystals. The diattenuation images obtained using Polarization microscopy were used to quantify for curvature of biocrystals using a ThreePointROI plugin for ImageJ, as used elsewhere[44]. Briefly, three points were marked along the maximum Feret's length of the high diattenuation signal of the crystal which was used by the plugin to draw a

circle through the three points and accordingly provide a radius of the circle (r). Curvature for this circle is then defined as $\kappa = 1/r$. Co-linear points that define an impossible circle resulted in a circle with r = "-1" or "2147483647" pixels and were marked as $\kappa=0$. A line was then drawn through the three points used to generate the circle to generate a chord of length (x) for that circle. Using the length of the chord and the radius, the angle formed by the biocrystal arc through that circle was then computed using the formula – $\theta = 2\sin^{-1}(x/2r)$. The arc length of the biocrystal was then computed as $L = \theta \times r$ where θ is in radians. Linear curvature density was computed as κ/L.

Synthesis of CFZ-HCl Crystals. To grow the biomimetic crystals (CFZ-HCl) in bulk, HCl was added to a 2mM CFZ in methanol solution until the HCl concentration was 0.1M. After the solution sat for 5 minutes, water is added to the solution to double the solution volume. Within minutes, thin dark red crystals are observed. To grow diffraction quality crystals of CFZ-HCl, 2mM CFZ was dissolved in benzene and 0.1M HCl was added to the solution. The solution was allowed to slowly evaporate and red, rectangular plate-like crystals were observed.

Qualitative Bending/Flexibility Analysis. Crystals that were about 2 mm long and ~20 μm thick were isolated from the crystallization solution and manipulated to demonstrate the crystal's elasticity. Crystals were placed on a microscope slide in a small amount of water to prevent the crystal from moving off the slide. A pair of tweezers and a crystallization probe were used to manipulate the crystals. Video of this procedure was taken with a Leica M205 C stereo microscope. See Movies 1 and 2. For analysis of bent crystals via polarization or Raman

microscopy, bent crystals were snap-frozen over dry ice and imaged either on a glass microscopy slide (Polarization) or a silicon wafer (Raman).

Powder X-Ray Diffraction (p-XRD). Powder XRD of isolated biocrystals was carried out as published before[25] with Bruker D8 Advance: Cu K_α radiation (λ = 1.5406 Å), tube voltage = 40 kV, and tube current = 40 mA. Data were collected at 2θ = 4° to 40° at a continuous scan rate of 2.5°/min. For CFZ-HCl crystals - data was collected on a Rigaku Miniflex 600 in the Bragg-Brentano geometry. The data was collected from 5° – 40°, 2θ with 0.02° steps and a 1.00 s detection time. Data was background subtracted using Origin® (Origin Labs, Northampton, MA)

Single Crystal X-Ray Diffraction. Single crystal X-ray data was collected on a Bruker D8 Venture equipped with a four-circle kappa diffractometer and Photon 100 detector with Cu source that supplied the multi-mirror monochromated incident beam. A combination of Phi and Omega scans were used to collect the necessary data. A single crystal was picked and mounted on a 0.3mm loop using paratone oil then cooled to 100 K in a nitrogen supplied Oxford 700 Cryostream. Data was integrated using SAINT and absorption corrected using SAINT/SADABS v2014/4. The final structure was solved using SHELX-2014-6.

Raman Microscopy. Confocal Raman microscopy was performed using a WITec alpha300 R equipped with a near-IR 785 nm to minimize clofazimine's fluorescence signal. Samples were positioned on the stage for spectral data acquisition and were observed using the reflectance illumination mode of the microscope. Once positioned, the 4 μm diameter 785 nm laser illumination spot was directed to the sample, and the Raman spectrum was acquired. Raw data

were background subtracted from the signal obtained from pure silicon wafers and further baseline-corrected using Origin® (Origin Labs, Northampton, MA).

Differential Scanning Calorimetry (DSC). Samples were analyzed using a TA Instruments 2910 MDSC system equipped with a refrigerated cooling unit. All experiments were performed by heating the pre-weighed samples at a rate of 10 °C/min under a dry nitrogen atmosphere. Temperature and enthalpy of the instrument were calibrated using high purity indium standard.

Supplementary Figures

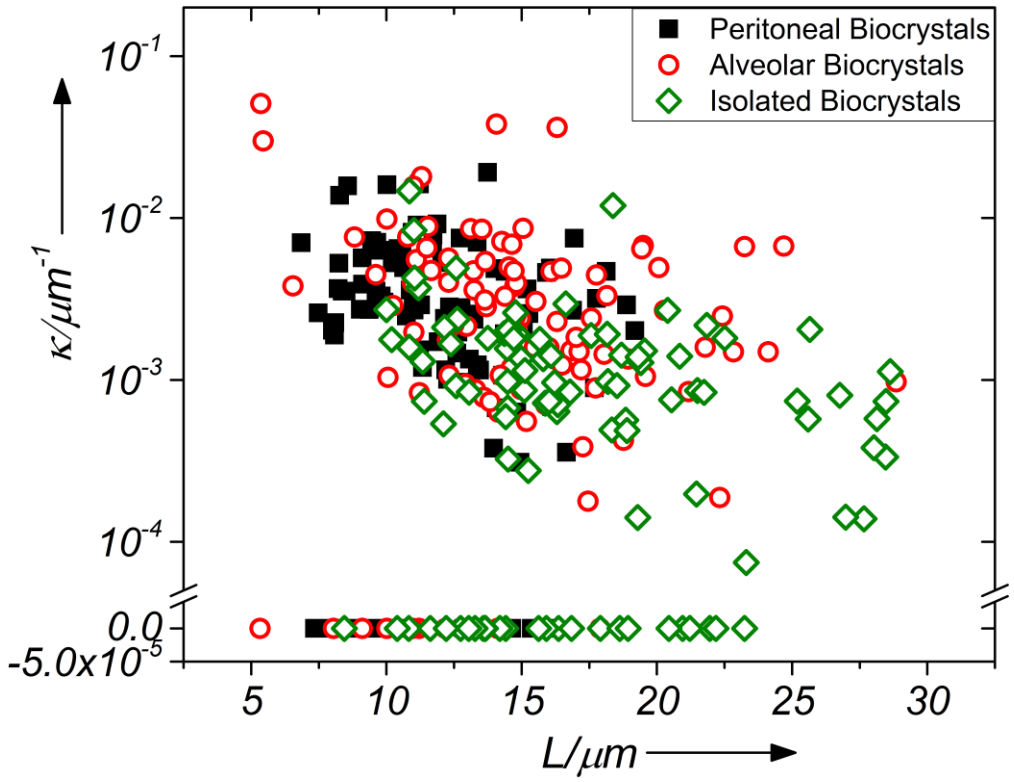

Figure S1. Curvature (κ) of biocrystals measured from two different macrophage populations (peritoneal and alveolar) and isolated biocrystals from the spleen plotted as a function of their arc length (L).

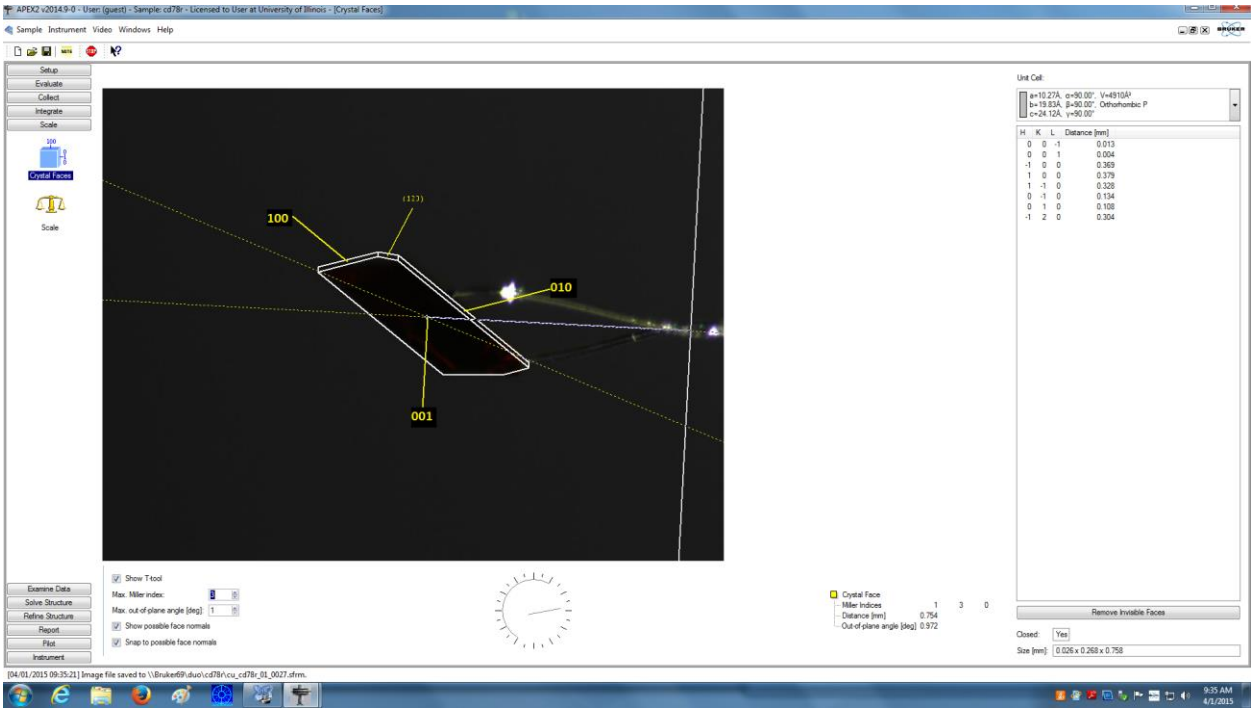

Figure S2. Face indexed image of CFZ-HCl crystal that was used for single crystals X-ray diffraction.

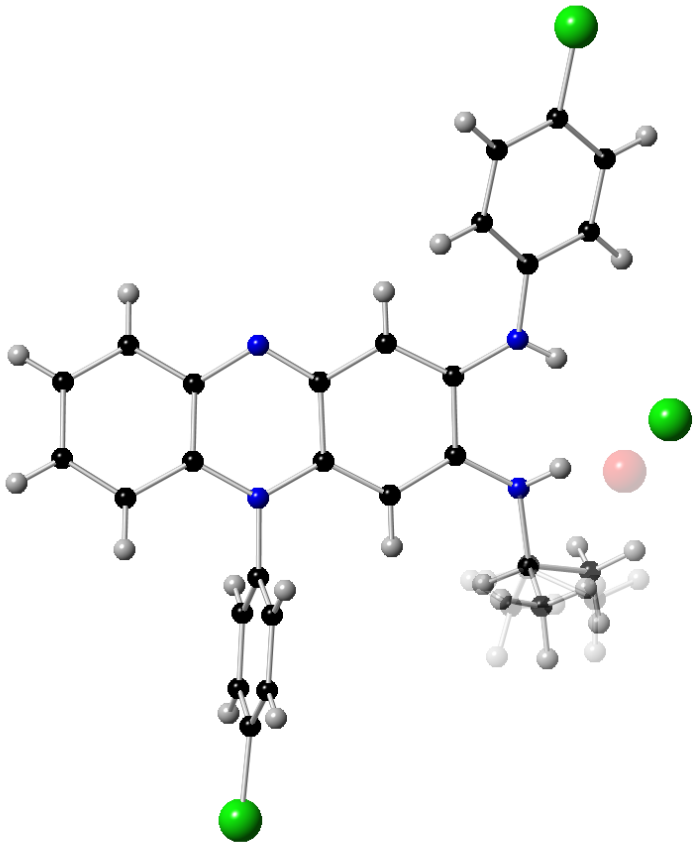

Figure S3. Asymmetric unit of CFZ-HCl including the position of the water molecule and the disorder in the propyl group showed as a transparent overlay. The water has ~13% occupancy and the presence of water is likely due to the solvent used in the crystallization solution or HCl.

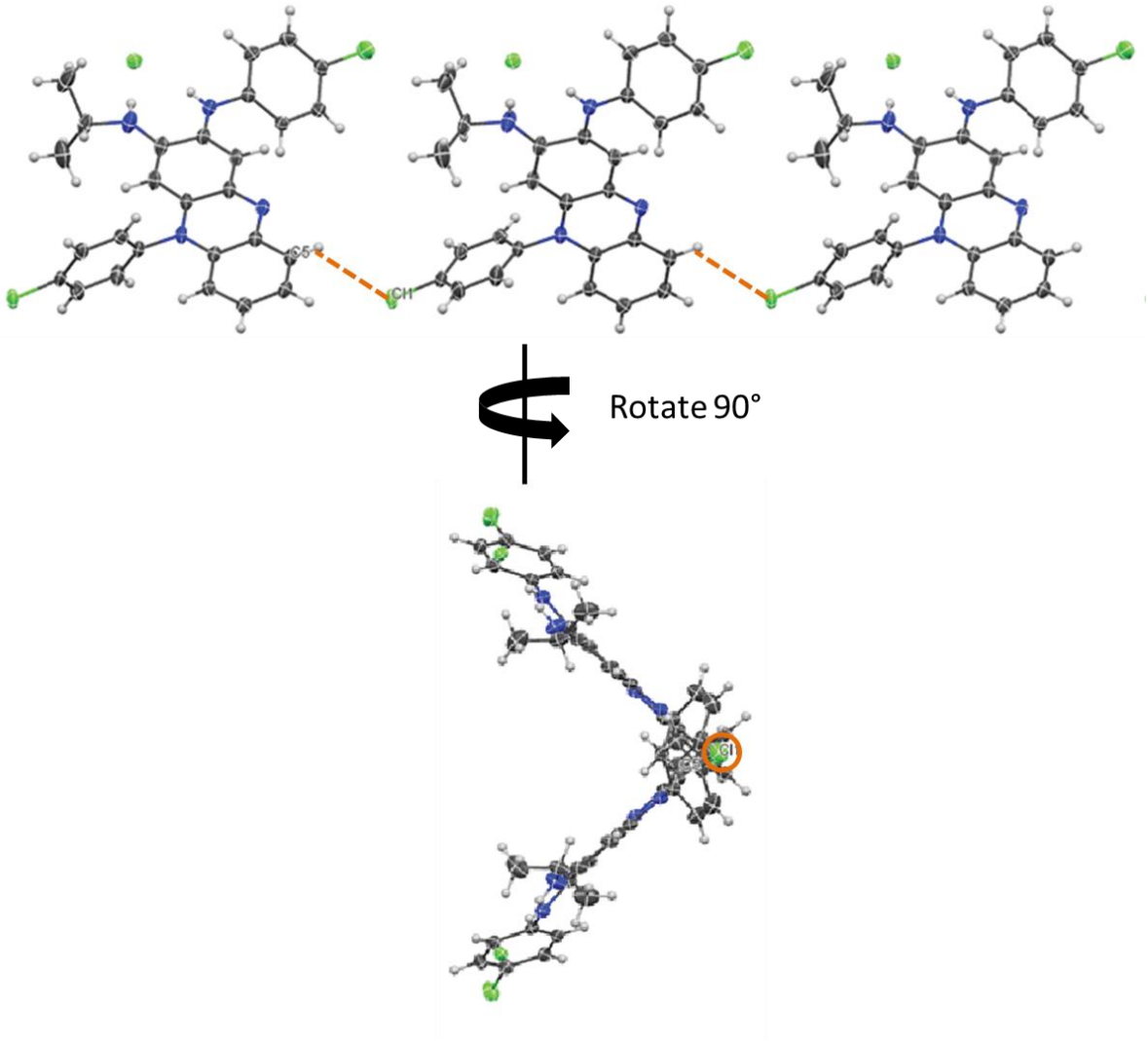

Figure S4. Cl1 interaction with C5-H5 viewed along the b-axis to form a chain of CFZ-HCl molecules and along the c-axis to show the position of the Cl1 interaction within the zig-zag.

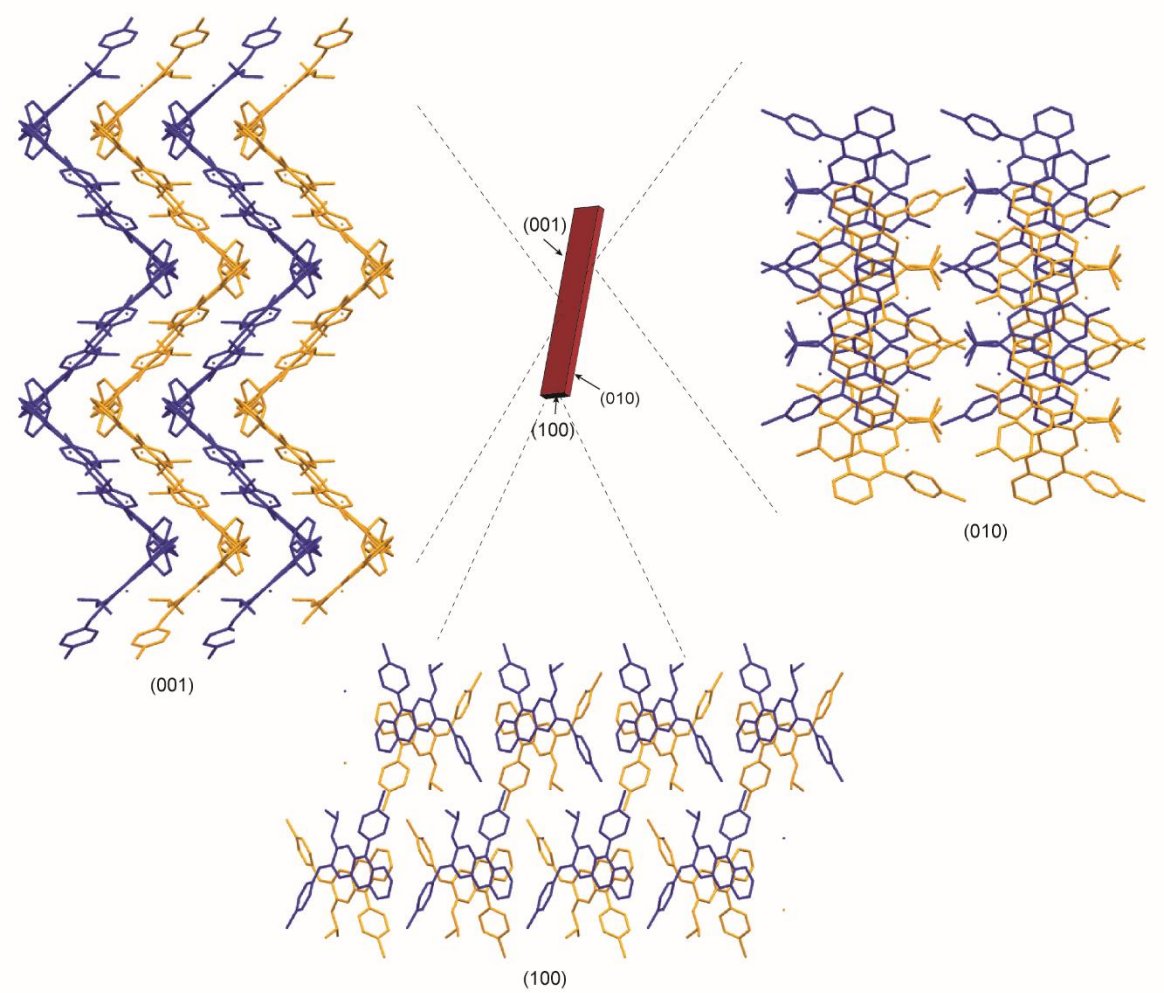

Figure S5. Crystallographic projection of the major faces of the CFZ-HCl crystal. The rows of corrugated structure along the (001) face are colored blue and orange to help visualize the crystal packing. The projections show a stacking of 2 x 2 x 2 (a x b x c) unit cells.

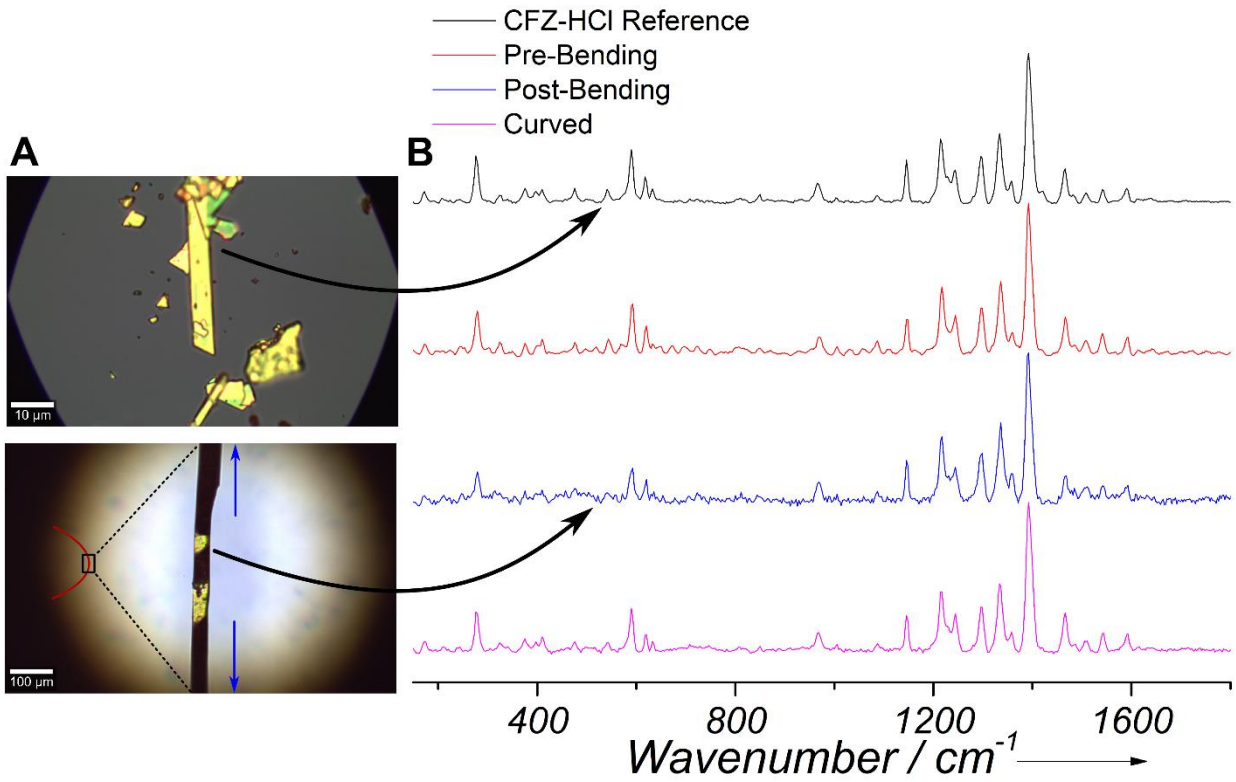

Figure S6. (A) Raman Reflectance Brightfield images of (*top*) short CFZ-HCl crystals and (*bottom*) at the bent point of a long CFZ-HCl elastic crystal. Blue arrows indicate moving away from the confocal plane used to obtain the point spectra of CFZ-HCl; (B) Raman Spectra of CFZ-HCl reference crystals *(top)* with crystals that were subjected to mechanical bending (before and after) *(second and third from top)* and crystals that had an inherent curvature *(bottom)*.

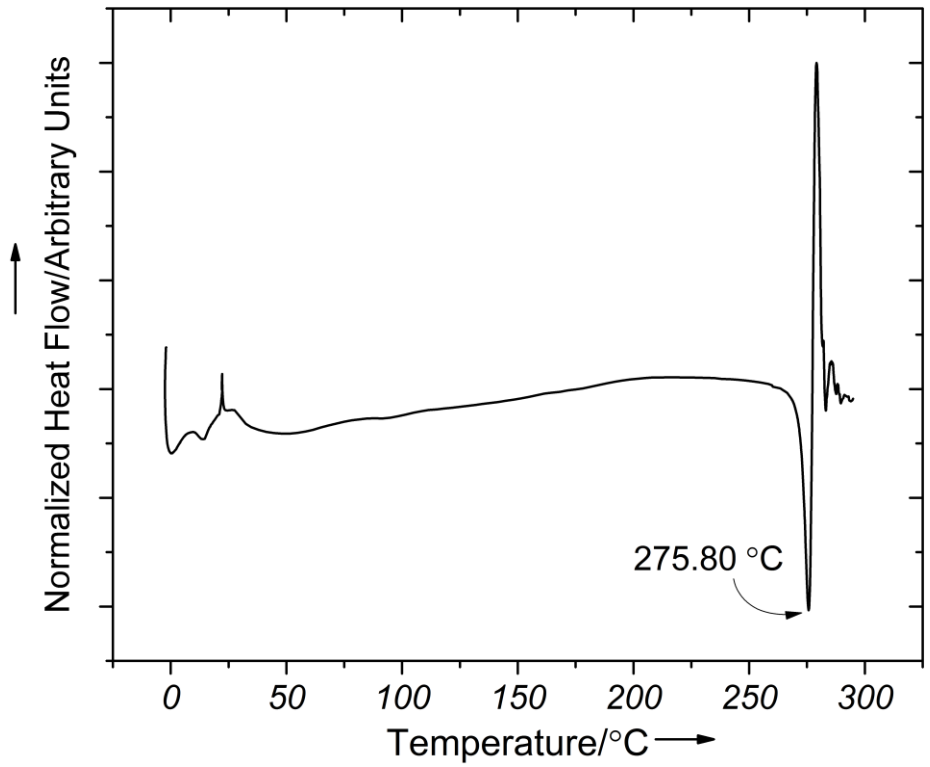

Figure S7. Differential Scanning Calorimetry of CFZ-HCl. Melting Point indicated with curved arrow – 275.80 °C.

Supplementary Tables

Table S1. Crystallographic data for CFZ-HCl.

Empirical formula	$C_{27}H_{23.26}Cl_3N_4O_{0.13}$
Formula weight (mg/ml)	512.18
Temperature (K)	100
Wavelength (Å)	1.54178
Crystal system	Orthorhombic
Space group	Pbca
a (Å)	10.266
b (Å)	19.828
c (Å)	24.156
α (°)	90
β (°)	90
ϒ (°)	90
Volume ($Å^3$)	4917.1
Z	8
Density (calc., mg/m^3)	1.384
Absorption coefficient (mm^{-1})	3.562
F(000)	2122
Crystal size (mm^3)	0.758 x 0.269 x 0.014
Theta range for data collection (°)	3.659 to 68.333
Index ranges	-12<=h<=12 -23<=k<=21 -29<=l<=29
Reflections collected	28160
Independent reflections	4500 [R(int) = 0.0352]
Completeness to theta max	99.8 %
Absorption correction	Integration
Max. and min. transmission	0.9615 and 0.3840
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	4500 / 134 / 365
Goodness-of-fit on F^2	1.069
Final R indices [I>2sigma(I)]	R1 = 0.0392, wR2 = 0.0982
R indices (all data)	R1 = 0.0422, wR2 = 0.1010
Extinction coefficient	0.00261
Largest diff. peak and hole ($e.Å^{-3}$)	0.685 and -0.415

Table S2. Molecular interactions within the CFZ-HCl crystal structure.

	D-H…A	D	d	theta
N-H···Cl	N3-H3A...Cl3	3.172	2.376	166.45
	N4-H4A...Cl3	3.104	2.243	174.51
C-H···Cl	C5-H5…Cl1	3.516	2.937	120.48
	C14-H14…Cl3	3.503	2.724	139.72
	C24-H24…Cl3	3.457	2.757	131.19
	C25_a-H25_a…Cl1	3.891	2.972	153.33
	C27_a-H27A_a…Cl3	3.671	2.899	136.36
π··· π	C1-C6…C7-C12	3.573		
	C1-C6…C1-N1-C12-C7-N2-C6	3.888		
	C19-C24…C7-C12	4.117		
H··· π	C3-H3…C19-C24	3.153		
	C21-H21…C1-C6	3.313		

with water present new interactions are formed

	D-H…A	D	d	theta
C-H···O	C17-H17...O1_b	3.076	2.583	112.62
	C18-H18...O1_b	3.005	2.442	117.82
C-H···Cl	C26B_b-H26E_b…Cl2	3.641	2.676	168.28
	C26B_b-H26F_b…Cl3	3.607	2.985	122.52
	C27B_b-H27F_b…Cl2	3.643	2.672	170.96

checkCIF (basic structural check) running

Checking for embedded fcf data in CIF ...
Found embedded fcf data in CIF. Extracting fcf data from uploaded CIF, please wait **...**

checkCIF/PLATON (basic structural check)

Structure factors have been supplied for datablock(s) cd78rsa

THIS REPORT IS FOR GUIDANCE ONLY. IF USED AS PART OF A REVIEW PROCEDURE FOR PUBLICATION, IT SHOULD NOT REPLACE THE EXPERTISE OF AN EXPERIENCED CRYSTALLOGRAPHIC REFEREE.

No syntax errors found. CIF dictionary
Please wait while processing .... Interpreting this report

Structure factor report

Datablock: cd78rsa

Bond precision: C-C = 0.0025 A Wavelength=1.54178

Cell: a=10.2664(4) b=19.8275(7) c=24.1558(9)
alpha=90 beta=90 gamma=90

Temperature: 100 K

	Calculated	Reported
Volume	4917.1(3)	4917.1(3)
Space group	P b c a	P b c a
Hall group	-P 2ac 2ab	-P 2ac 2ab
Moiety formula	C27 H23 Cl2 N4, Cl, 0.128(O)	C27 H23 Cl2 N4, Cl, 0.13(O H2)
Sum formula	C27 H23 Cl3 N4 O0.13	C27 H23.26 Cl3 N4 O0.13
Mr	511.89	512.18
Dx,g cm-3	1.383	1.384
Z	8	8
Mu (mm-1)	3.562	3.562
F000	2120.2	2122.0
F000'	2133.63	
h,k,lmax	12,23,29	12,23,29
Nref	4512	4500
Tmin,Tmax	0.364,0.951	0.384,0.962
Tmin'	0.058	

Correction method= # Reported T Limits: Tmin=0.384 Tmax=0.962 AbsCorr = INTEGRATION

Data completeness= 0.997 Theta(max)= 68.333

R(reflections)= 0.0392(4149) wR2(reflections)= 0.1010(4500)

S = 1.069 Npar= 365

The following ALERTS were generated. Each ALERT has the format
test-name_ALERT_alert-type_alert-level.
Click on the hyperlinks for more details of the test.

Alert level C

PLAT041_ALERT_1_C Calc. and Reported SumFormula Strings Differ Please Check
PLAT068_ALERT_1_C Reported F000 Differs from Calcd (or Missing)... Please Check

PLAT077_ALERT_4_C Unitcell contains non-integer number of atoms .. Please Check
PLAT410_ALERT_2_C Short Intra H...H Contact H11 .. H25B .. 1.99 Ang.
PLAT480_ALERT_4_C Long H...A H-Bond Reported H5 .. CL1 .. 2.94 Ang.
And 4 other PLAT480 Alerts
More ...
PLAT911_ALERT_3_C Missing # FCF Refl Between THmin & STh/L= 0.600 9 Report

Alert level G

FORMU01_ALERT_2_G There is a discrepancy between the atom counts in the
_chemical_formula_sum and the formula from the _atom_site* data.
Atom count from _chemical_formula_sum:C27 H23.26 Cl3 N4 O0.13
Atom count from the _atom_site data: C27 H23 Cl3 N4 O0.128
CELLZ01_ALERT_1_G Difference between formula and atom_site contents detected.
CELLZ01_ALERT_1_G WARNING: H atoms missing from atom site list. Is this intentional?
From the CIF: _cell_formula_units_Z 8
From the CIF: _chemical_formula_sum C27 H23.26 Cl3 N4 O0.13
TEST: Compare cell contents of formula and atom_site data

atom	Z*formula	cif sites	diff
C	216.00	216.00	0.00
H	186.08	184.00	2.08
Cl	24.00	24.00	0.00
N	32.00	32.00	0.00
O	1.04	1.02	0.02

PLAT002_ALERT_2_G Number of Distance or Angle Restraints on AtSite 11 Note
PLAT003_ALERT_2_G Number of Uiso or Uij Restrained non-H Atoms ... 8 Report
PLAT042_ALERT_1_G Calc. and Reported MoietyFormula Strings Differ Please Check
PLAT063_ALERT_4_G Crystal Size Likely too Large for Beam Size .... 0.76 mm
PLAT172_ALERT_4_G The CIF-Embedded .res File Contains DFIX Records 1 Report
PLAT175_ALERT_4_G The CIF-Embedded .res File Contains SAME Records 1 Report
PLAT176_ALERT_4_G The CIF-Embedded .res File Contains SADI Records 2 Report
PLAT178_ALERT_4_G The CIF-Embedded .res File Contains SIMU Records 2 Report
PLAT186_ALERT_4_G The CIF-Embedded .res File Contains ISOR Records 2 Report
PLAT301_ALERT_3_G Main Residue Disorder .....(Resd 1).. 12 % Note
PLAT302_ALERT_4_G Anion/Solvent/Minor-Residue Disorder (Resd 3).. 100 % Note
PLAT304_ALERT_4_G Non-Integer Number of Atoms (0.13) in Resd. # 3 Check
PLAT311_ALERT_2_G Isolated Disordered Oxygen Atom (No H's ?) ..... O1 Check
PLAT333_ALERT_2_G Check Large Av C6-Ring C-C Dist. C7 -C12 1.42 Ang.
PLAT432_ALERT_2_G Short Inter X...Y Contact O1 .. C18 .. 3.01 Ang.
PLAT860_ALERT_3_G Number of Least-Squares Restraints ..... 134 Note
PLAT912_ALERT_4_G Missing # of FCF Reflections Above STh/L= 0.600 3 Note
PLAT913_ALERT_3_G Missing # of Very Strong Reflections in FCF .... 2 Note
PLAT933_ALERT_2_G Number of OMIT Records in Embedded .res File ... 1 Note
PLAT978_ALERT_2_G Number C-C Bonds with Positive Residual Density. 12 Note

0 **ALERT level A** = Most likely a serious problem - resolve or explain
0 **ALERT level B** = A potentially serious problem, consider carefully
10 **ALERT level C** = Check. Ensure it is not caused by an omission or oversight
23 **ALERT level G** = General information/check it is not something unexpected

5 ALERT type 1 CIF construction/syntax error, inconsistent or missing data
9 ALERT type 2 Indicator that the structure model may be wrong or deficient
4 ALERT type 3 Indicator that the structure quality may be low
15 ALERT type 4 Improvement, methodology, query or suggestion
0 ALERT type 5 Informative message, check

It is advisable to attempt to resolve as many as possible of the alerts in all categories. Often the minor alerts point to easily fixed oversights, errors and omissions in your CIF or refinement strategy, so attention to these fine details can be worthwhile. In order to resolve some of the more serious problems it may be necessary to carry out additional measurements or structure refinements. However, the purpose of your study may justify the reported deviations and the more serious of these should normally be commented upon in the discussion or experimental section of a paper or in the "special_details" fields of the CIF. checkCIF was carefully designed to identify outliers and unusual parameters, but every test has its limitations and alerts that are not important in a particular case may appear. Conversely, the absence of alerts does not guarantee there are no aspects of

the results needing attention. It is up to the individual to critically assess their own results and, if necessary, seek expert advice.

Publication of your CIF in IUCr journals

A basic structural check has been run on your CIF. These basic checks will be run on all CIFs submitted for publication in IUCr journals (*Acta Crystallographica*, *Journal of Applied Crystallography*, *Journal of Synchrotron Radiation*); however, if you intend to submit to *Acta Crystallographica Section C* or *E* or *IUCrData*, you should make sure that full publication checks are run on the final version of your CIF prior to submission.

Publication of your CIF in other journals

Please refer to the *Notes for Authors* of the relevant journal for any special instructions relating to CIF submission.

PLATON version of 24/11/2016; check.def file version of 23/11/2016

Datablock cd78rsa - ellipsoid plot