Supporting information

IN-CELL QUANTIFICATION OF DRUGS BY MAGIC ANGLE SPINNING DYNAMIC NUCLEAR POLARIZATION NMR

Andrea Bertarello,[1] Pierrick Berruyer,[1] Markus Artelsmair,[2] Charles S. Elmore,[2] Sepideh Heydarkhan-Hagvall,[3] Markus Schade,[4] Elisabetta Chiarparin,[4] Staffan Schantz,[5,*] Lyndon Emsley[1,*]

[1] Institut des Sciences et Ingénierie Chimiques, École Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland

[2] Early Chemical Development, Pharmaceutical Science, R&D, AstraZeneca, SE-431 83 Mölndal, Sweden

[3] Bioscience, Research and Early Development, Cardiovascular, Renal and Metabolism (CVRM), BioPharmaceutical R&D Astra-Zeneca, SE-431 83 Mölndal, Sweden, Sweden

[4] Chemistry, Oncology R&D, AstraZeneca, Cambridge CB4 0WG, United Kingdom

[5] Oral Product Development, Pharmaceutical Technology & Development, Operations, AstraZeneca, SE-431 83 Mölndal, Sweden

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Raw data statement

The NMR raw data are available (DOI: 10.5281/zenodo.5993290) in the original TopSpin and JCAMP formats. Data are made available under the license CC-BY-4.0 (http://creativecommons.org/licenses/by/4.0/ Creative Commons Attribution 4.0 International).

Additional experimental details

Synthesis of [^{15}N]CHIR-98014

General experimental

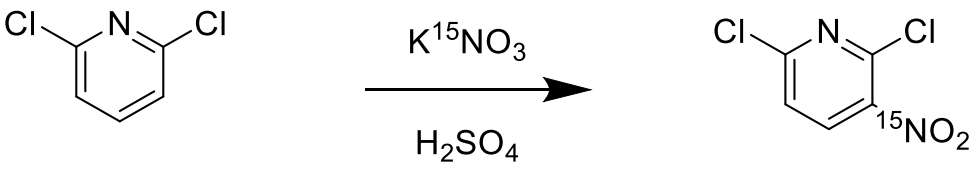
All reagents were purchased from commercial sources (Fluka, Sigma, and RP Normapur) and were used without further purification. $K^{15}NO_3$ was purchased from SigmaAldrich. N1-(4-(2,4-dichlorophenyl)-5-(1H-imidazol-1-yl)pyrimidin-2-yl)ethane-1,2-diamine was prepared by Key Organics (Cornwall, England) according to a published procedure. [1]

The identity of the synthesized compounds was determined by comparison of the LCMS or GCMS and NMR with authentic unlabeled material. LCMS was acquired on a HP 1100 system with 1100 series degasser, 1100 series pump, 1100 series column oven, and 1100 series diode array with a waters 2700 sample manager and Waters 3100 mass detector. Column conditions are: 5 to 100% MeCN-0.1% Formic acid (adjusted to pH 3) over 10 min followed by a 2 min wash with 100% MeCN. Column: Waters Xselect CSH C-18 4.6/150 mm, 3.5 micron.

GC–MS (EI) analysis was performed on a 7890A GC system and 5975C inert MSD system equipped with an Agilent 19091S-433L (30 m × 250 μ × 0.25 μm) capillary column using a gradient: 40–150 °C with a rate of 15 °C/min followed by 150–300 °C with a rate of 60 °C/min and electron impact ionization at 70 eV.

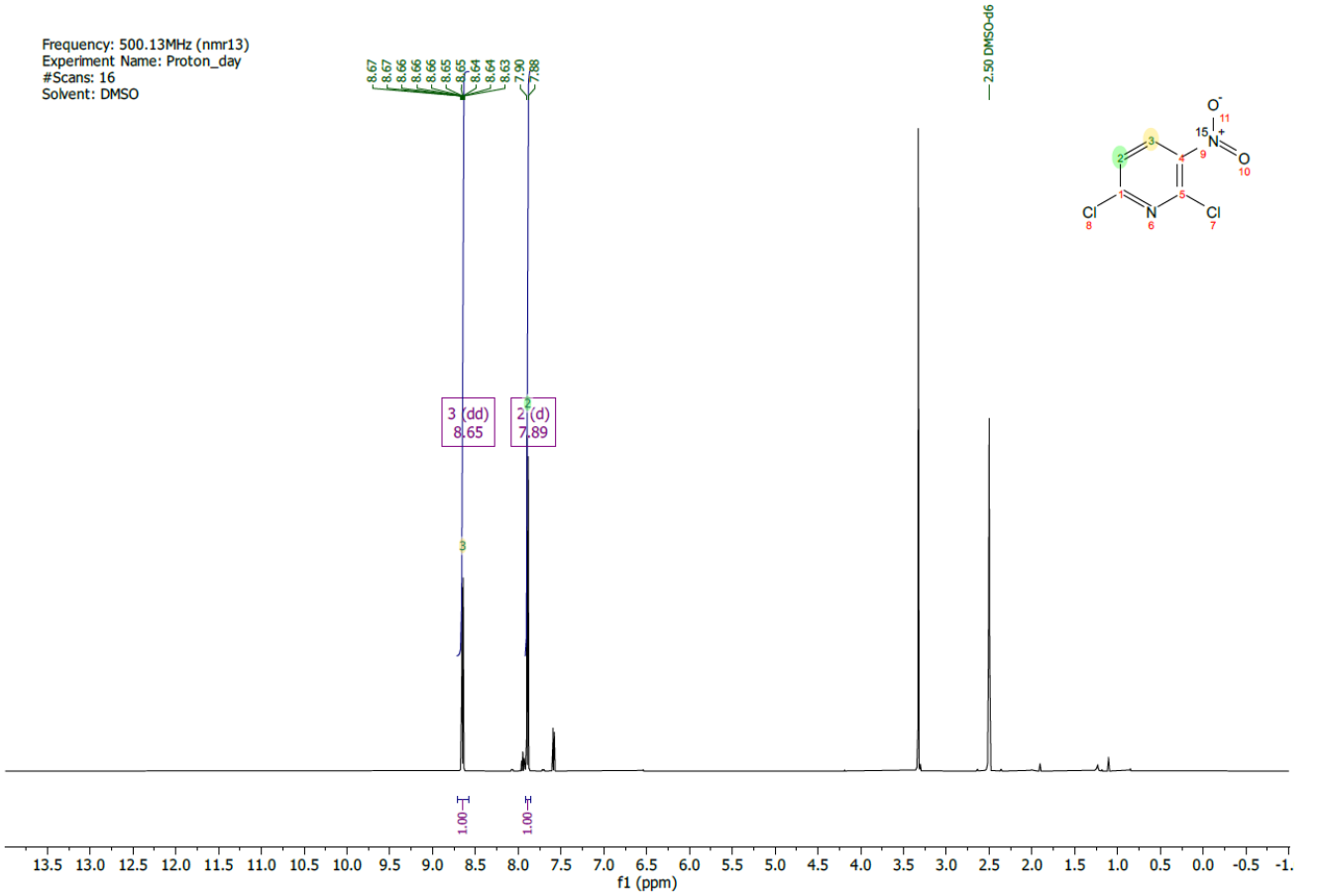
^{1}H NMR, ^{13}C and ^{15}N NMR spectra were recorded on a Bruker AVANCE III system running at a proton frequency of 500.1 MHz with a cryogenic probe and processed with the NMR software MestreNova (Mestrelab Research SL). NMR experiments were run in DMSO-d_6 at 25 °C, unless stated otherwise. ^{1}H chemical shifts are referenced relative to the residual solvent peak at 2.50 ppm, and ^{13}C chemical shifts are referenced to 39.52 ppm for DMSO. Signals are listed in ppm, multiplicity (identified as s = singlet, bs = broad singlet, d = doublet, dd = doublet of a doublet, t = triplet, tt = triplet of a triplet, q = quartet, and m = multiplet; coupling constants in Hz) and integration. ^{13}C NMR data is reported as chemical shifts and multiplicity (coupling constants in Hz) where coupling to ^{15}N is observed.

The synthesis of 2,6-dichloro-3-([^{15}N]nitro)pyridine

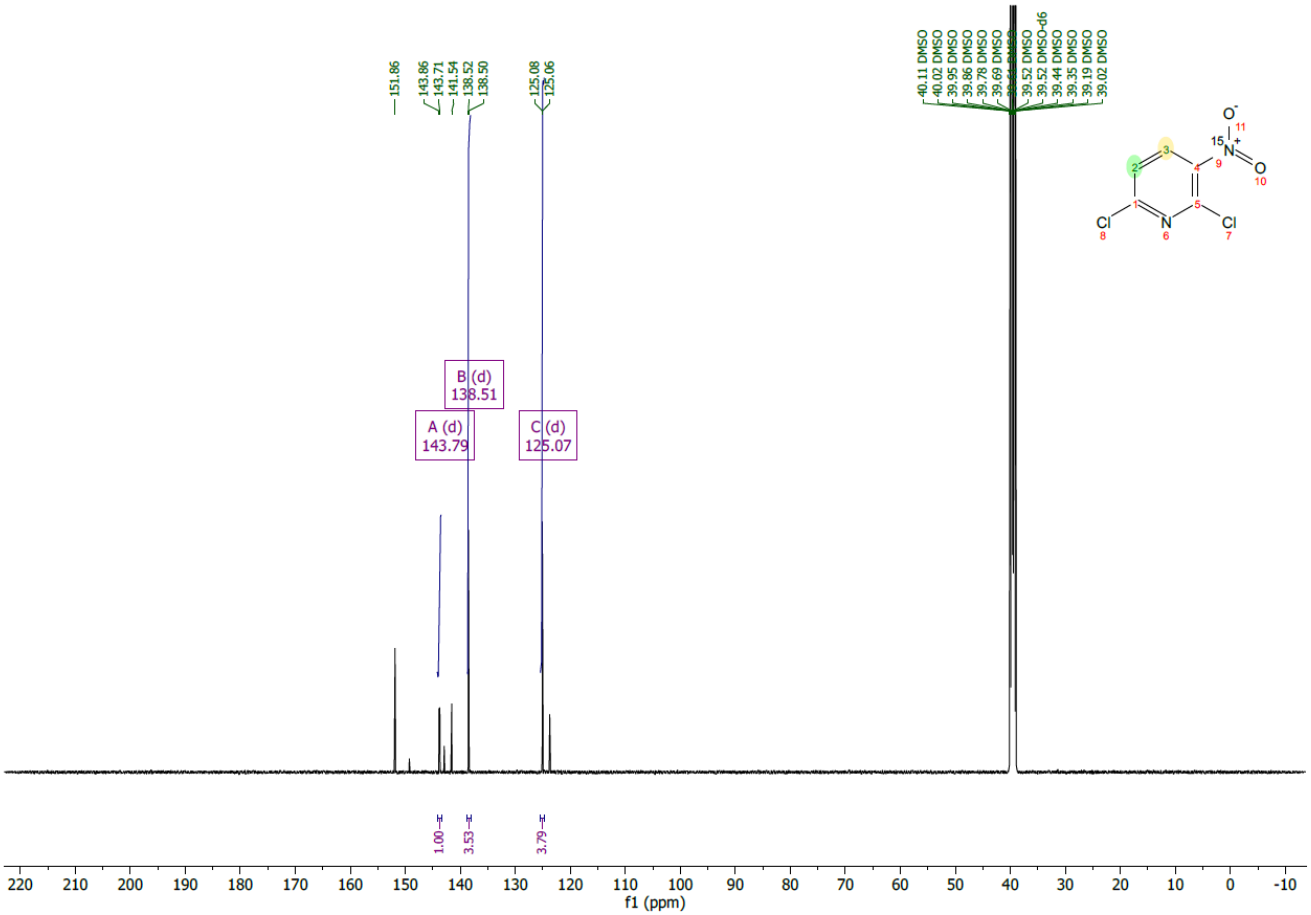


This preparation uses an adaptation of a literature procedure.[2]

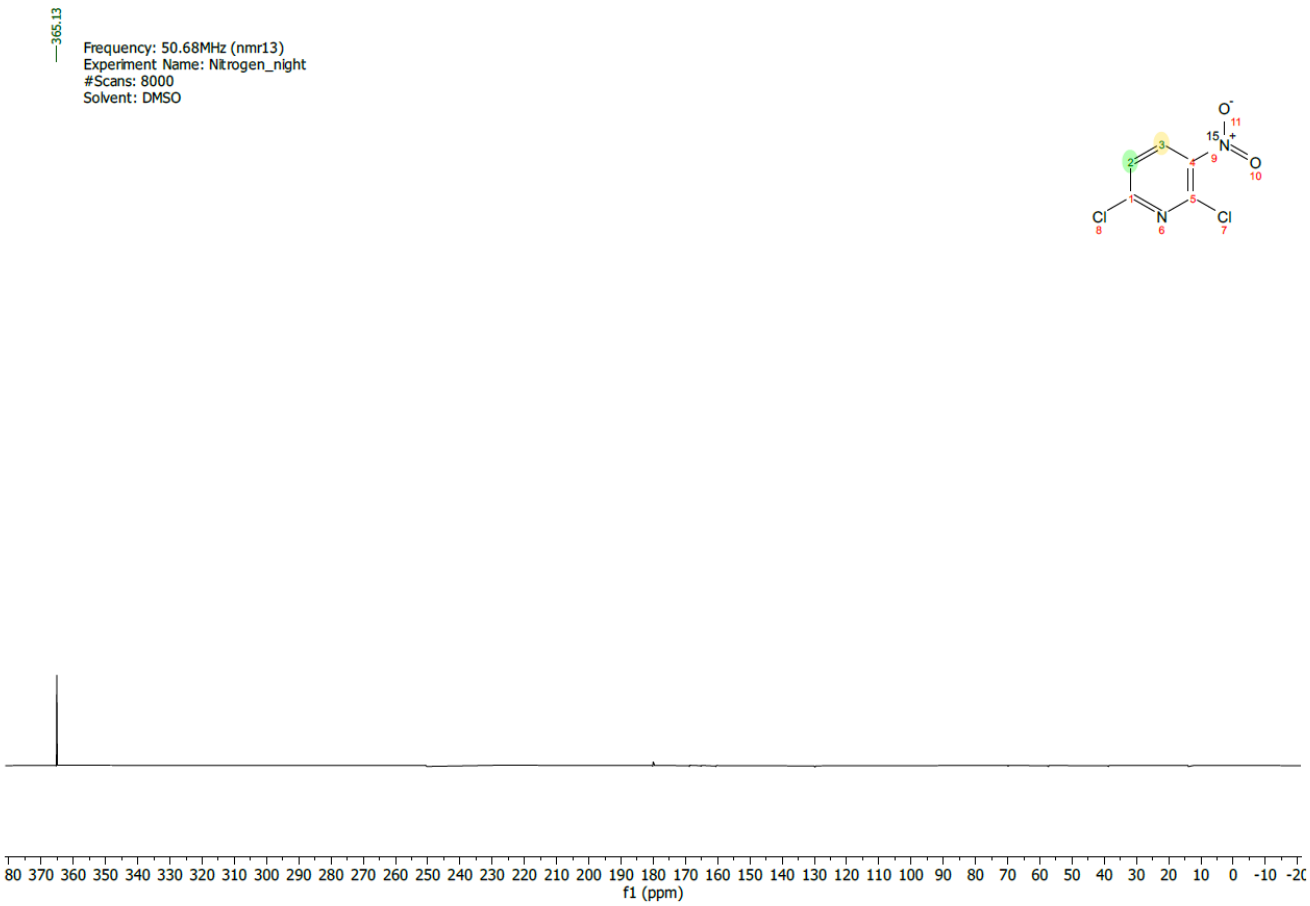
A solution of 2,6-dichloropyridine (1150 mg, 7.78 mmol) in H_2SO_4 (10 mL) was stirred at room temperature as $K^{15}NO_3$ (1870 mg, 18.3 mmol) was added in three portions over 10 min. After 10 min of vigorous stirring at room temperature, the slightly turbid solution clarified. The solution was warmed to 70 °C for 1 h and then to 120 °C for 18 h. After cooling to room temperature, the solution was added slowly to ice, and after the ice melted, was filtered through a glass frit to give a 798 mg (4.11 mmol, 53%) of a white solid. LCUV and NMR show a 9:1 ratio of 2,6-dichloro-3-([^{15}N]nitro)pyridine : 2.6-dichloropyridine.



^{1}H NMR (500 MHz, DMSO-d_6) δ 7.89 (d, J = 8.5 Hz, ^{1}H), 8.65 (dd, J = 8.4, 2.3 Hz, ^{1}H).



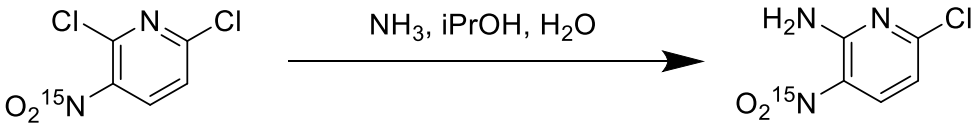
^{13}C NMR (126 MHz, DMSO-d_6) δ 151.86, 143.79 (d, J = 18.6 Hz), 141.54, 138.51 (d, J = 2.0 Hz), 125.07 (d, J = 1.8 Hz).



^{15}N NMR (51 MHz, DMSO-d_6) δ 365.13.

GC–MS: 193.0 [M], rt 4.98 min.

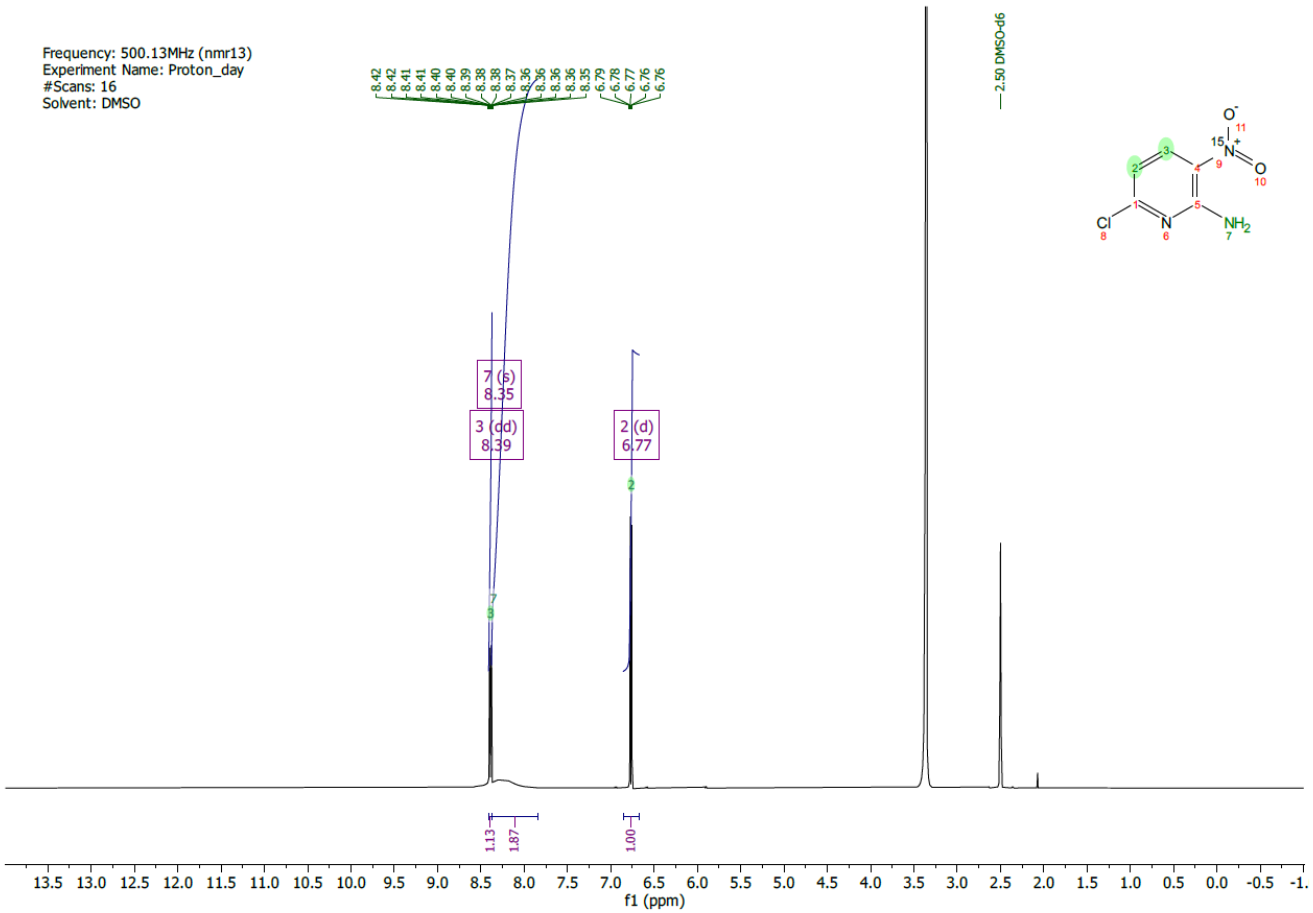
The synthesis of 6-chloro-3-([^{15}N]nitro)pyridin-2-amine



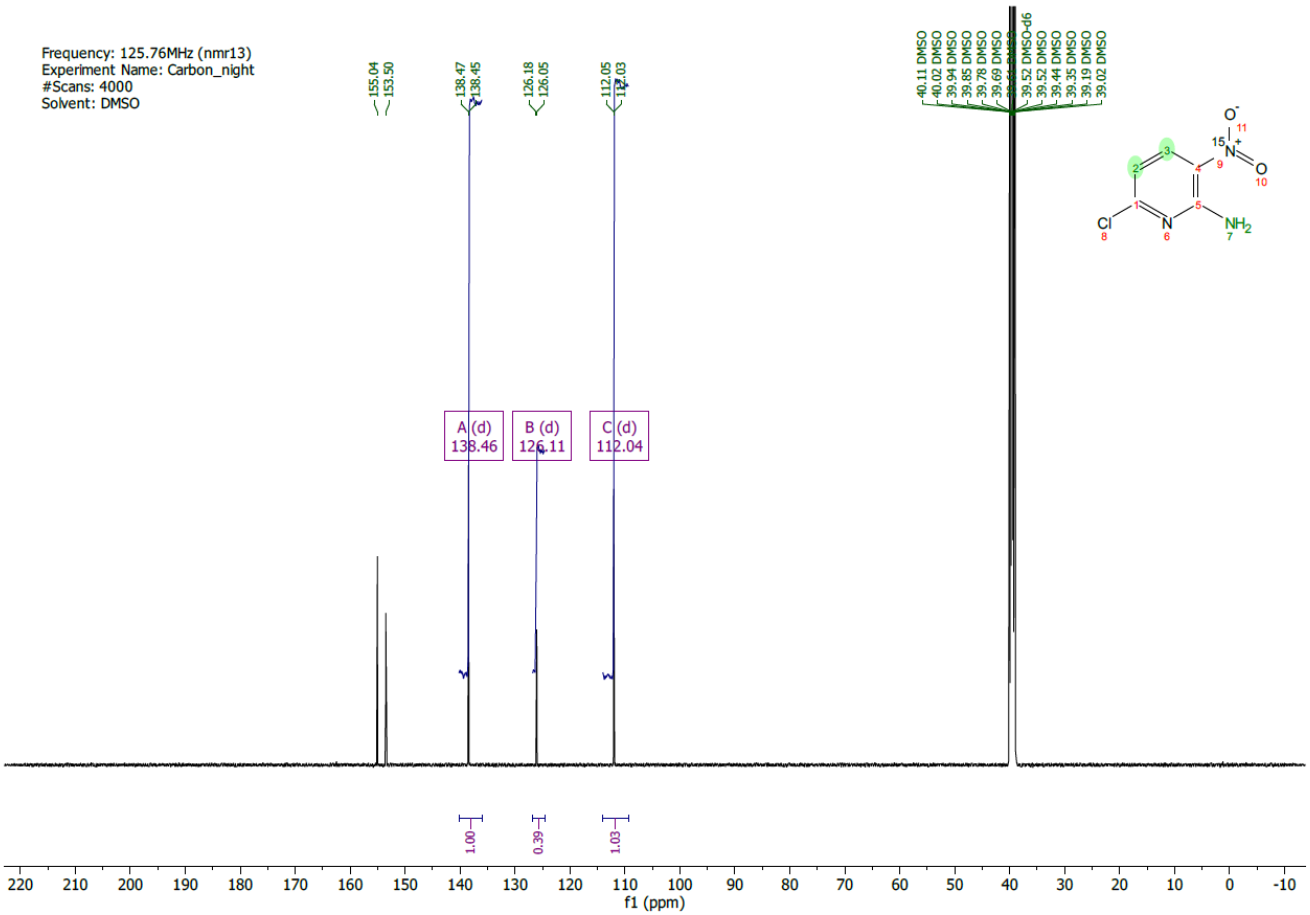
*safety note: This reaction was performed behind a blast shield

This preparation uses an adaptation of a literature procedure.[3]

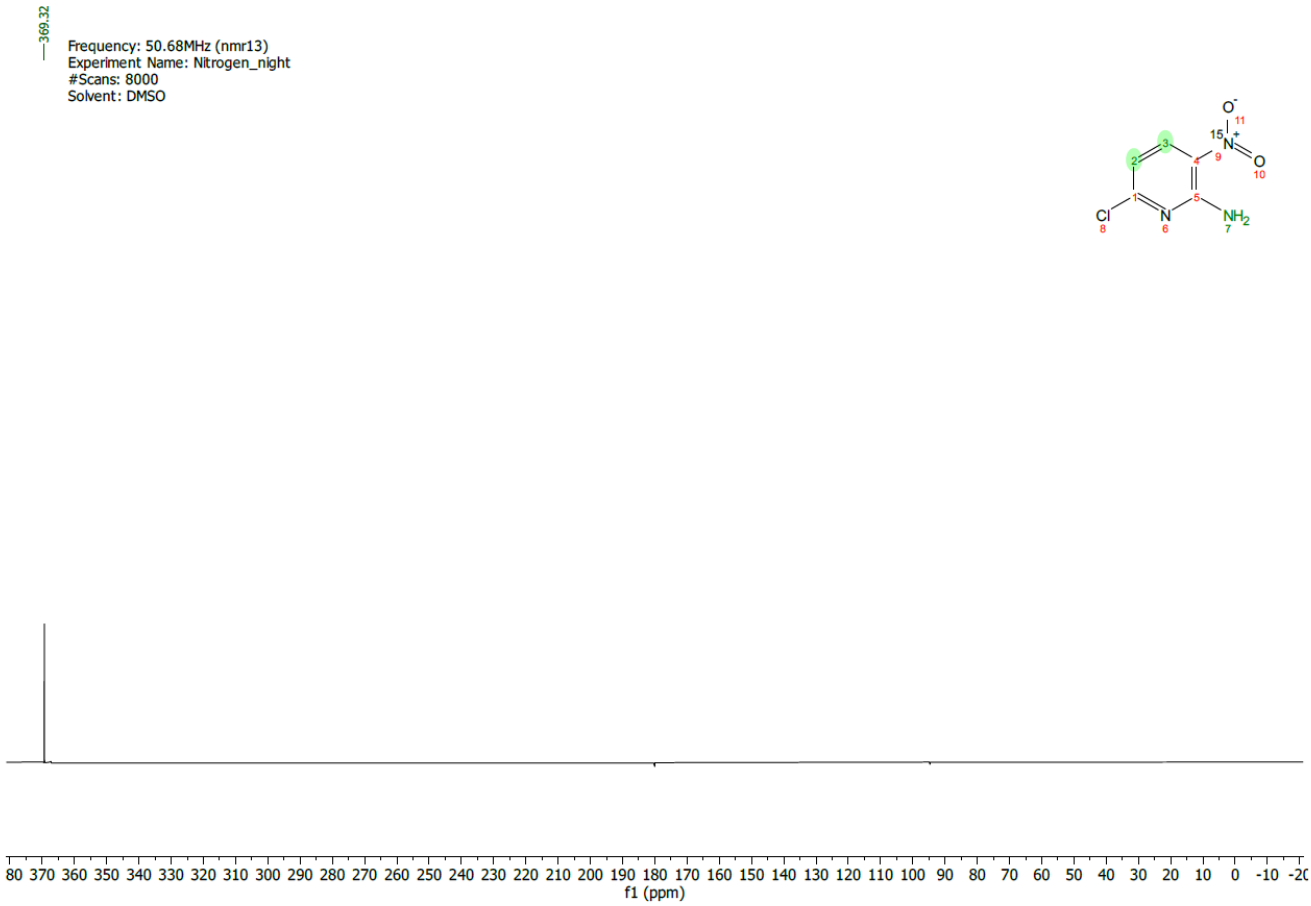
A solution of 2,6-dichloro-3-(nitro)pyridine (798 mg, 4.11 mmol, 90% UV purity) in 2-propanol (30 ml) and aq. NH_3 (3 mL, 78 mmol) in a sealed tube was stirred at room temperature for 30 min and at 35 °C for 3 days. The reaction was cooled to room temperature, and the solids were removed by filtration. The solids were washed with 20 mL of water and were then taken up in 5 mL of MeCN. The organic solution was concentrated to dryness to give 518 mg (2.97 mmol, 72%) of a yellow solid.



^{1}H NMR (500 MHz, DMSO-d_6) δ 6.77 (d, J = 8.6 Hz, 1H), 8.35 (bs, 2H), 8.39 (dd, J = 8.6, 2.1 Hz, 1H).



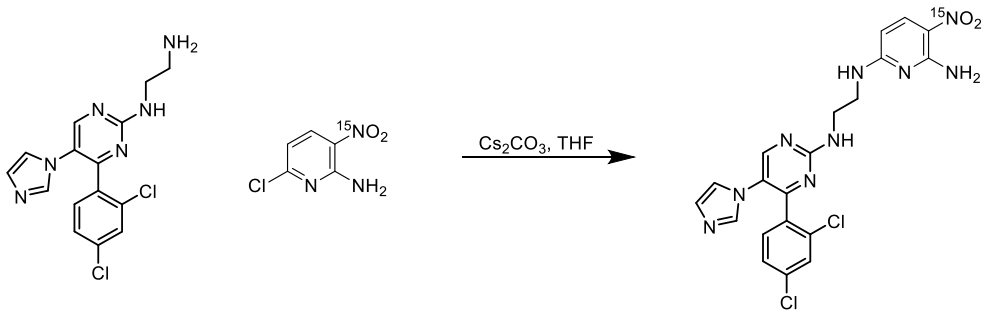
^{13}C NMR (126 MHz, DMSO-d_6) δ 155.04, 153.50, 138.46 (J = 1.8 Hz), 126.11 (J = 16.3 Hz), 112.04 (J = 2.1 Hz).



^{15}N NMR (51 MHz, DMSO-d_6) δ 369.32.

GC–MS: 174.0 [M], rt 5.40 min.

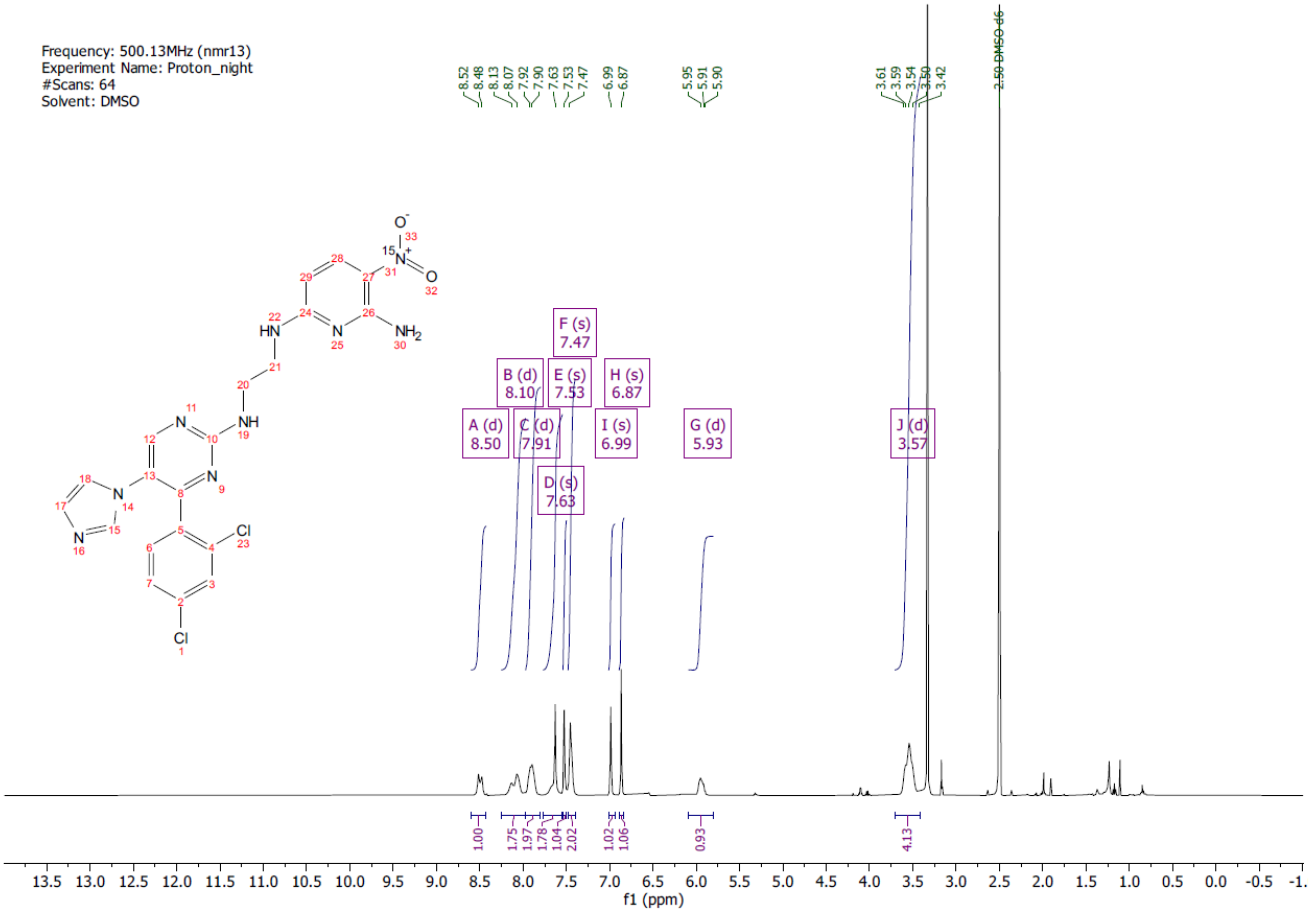
The synthesis of N2-(2-((4-(2,4-dichlorophenyl)-5-(1H-imidazol-1-yl)pyrimidin-2-yl)-amino)ethyl)-5-([^{15}N]nitro)pyridine-2,6-diamine



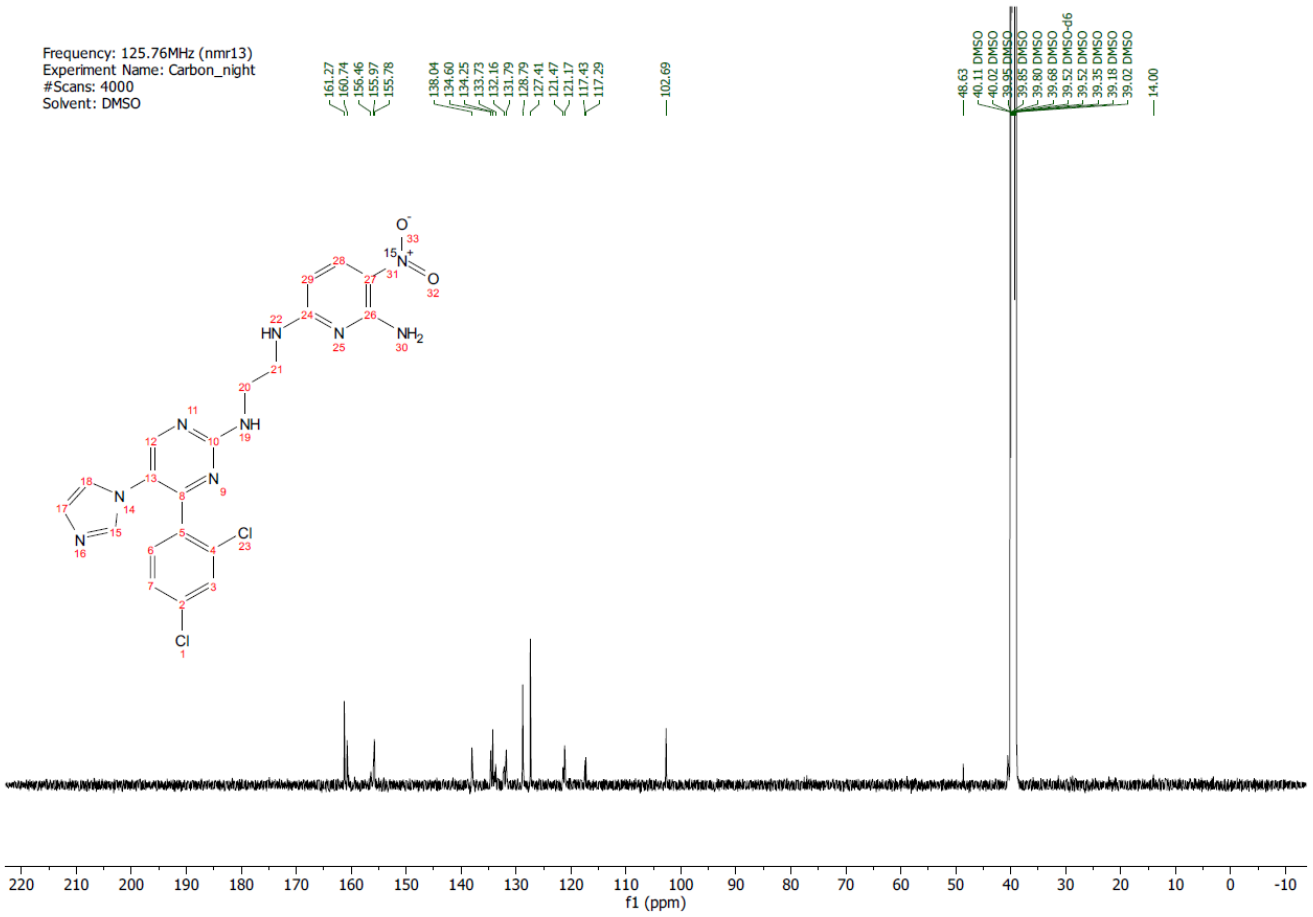
This preparation uses an adaptation of a literature procedure.[1]

A solution of N1-(4-(2,4-dichlorophenyl)-5-(1H-imidazol-1-yl)pyrimidin-2-yl)ethane-1,2-diamine (217 mg. 0.62 mmol), 6-chloro-3-([^{15}N]nitro)pyridin-2-amine (104 mg, 0.6 mmol), and Cs_2CO_3 (425 mg, 1.3 mmol) was stirred at 70 °C for 24 h under N_2. The reaction

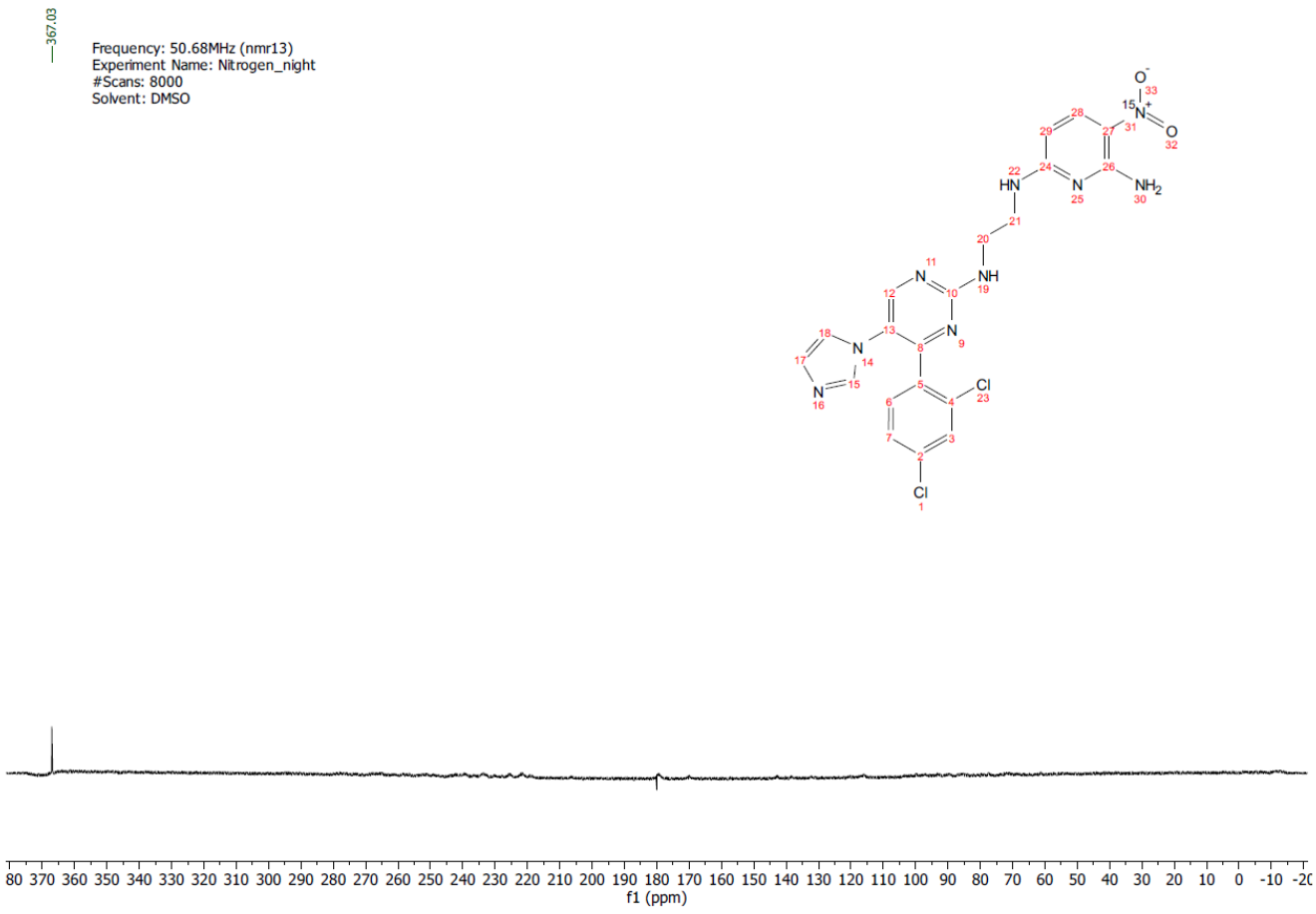
mixture was diluted with 40 mL of 2 M HCl and 50 mL of EtOAc and was vigorously agitated in a separatory funnel. The organic layer was removed, and the aqueous layer was washed with EtOAc (2x50 mL). The aqueous layer was basified to approximately pH 8 with 6 M NaOH and was extracted with EtOAc (3x50 mL). The combined organic layers were dried ($MgSO_4$) and filtered, and the resulting organic solution was concentrated to dryness. The resulting solids were stored under high vacuum to give a yellow solid (139 mg, 0.285 mmol, 47.9 %).



^{1}H NMR (500 MHz, DMSO-d_6) δ 3.57 (d, *J* = 24.0 Hz, 4H), 5.93 (d, *J* = 16.8 Hz, 1H), 6.87 (s, 1H), 6.99 (s, 1H), 7.47 (s, 2H), 7.53 (s, 1H), 7.63 (s, 2H), 7.91 (d, *J* = 11.9 Hz, 2H), 8.10 (d, *J* = 34.8 Hz, 2H), 8.50 (d, *J* = 18.8 Hz, 1H). *Note: Compound poorly soluble, broad peaks.*



^{13}C NMR (126 MHz, DMSO-d_6) δ 161.27, 160.74, 156.46, 155.97, 155.78, 138.04, 134.60, 134.25, 133.73, 132.16, 131.79, 128.79, 127.41, 121.47, 121.17, 117.43, 117.29, 102.69, 48.63, 14.00.



^{15}N NMR (51 MHz, DMSO-d_6) δ 367.03.

LC/MS (rt 5.4 min): M+1(intensity) 487.2 (100), 488.2 (26%), 489.2 (69%), 490.2 (16%), 491.2 (3%)

Additional tables

Table S1. Calculation of total aminoacid content in HEK cells and their contribution to the amide ^{15}N signal.

a. From reference [4]

b. Number of ^{15}N nuclei contributing to the NMR signal at ~120 ppm per residue.

c. Total amount of ^{15}N nuclei contributing to the NMR signal ~120 ppm.

Metabolite	pg/cell[a]	MM (g/mol)	N[b]	n (pmol/cell) [c]
Ala	32.1	89.09	1	0.36
Arg	20.2	174.20	1	0.12
Asn	15.4	132.12	2	0.23
Asp	19.2	133.10	1	0.14
Cys	7.8	121.16	1	0.06
Gln	17.2	146.14	2	0.24
Glu	20.6	147.13	1	0.14
Gly	28.8	75.07	1	0.38
His	7.6	155.15	1	0.05
Ile	17.3	131.17	1	0.13
leu	30.1	131.18	1	0.23
Lys	30.5	146.16	1	0.21
Met	7.4	149.21	1	0.05
Phe	11.7	165.19	1	0.07
Pro	16.7	115.13	1	0.15
Ser	23.0	105.09	1	0.22
Thr	20.6	119.12	1	0.17
Trp	2.4	204.23	2	0.02
Tyr	9.7	181.19	1	0.05
Val	22.2	117.15	1	0.19
SUM	360.5			3.22

Table S3. Relevant experimental parameters for (^{1}H)-^{15}N Multiple-contact CP of HEK cells incubated with CHIR-98014 5 μM (**1**) and CHIR-98014 10 μM (**2**), and (^{1}H)-^{13}C CP of HEK cells incubated with CHIR-98014 5 μM (**3**) and CHIR-98014 10 μM (**4**).

	1	**2**	**3**	**4**
MAS rate	10.5 kHz	13 kHz	10.5 kHz	13 kHz
Recycle delay	0.1 s	0.1s	3.5 s	4.25 s
Number of saturation pulses	15	15	15	15
Delay between saturation pulses	3 ms	3 ms	3 ms	3 ms
^{1}H ν_1 for pulses	100 kHz	100 kHz	100 kHz	100 kHz
^{13}C ν_1 for pulses	-	-	71 kHz	71 kHz
^{15}N ν_1 for pulses	50 kHz	50 kHz	-	-
^{1}H decoupling	SPINAL-64 (ν_1 = 100 kHz)	SPINAL-64 (ν_1 = 100 kHz)	SPINAL-64 (ν_1 = 100 kHz)	SPINAL-64 (ν_1 = 100 kHz)
τ_{CP}	5 ms	15 ms	500 μs	1 ms
^{1}H ν_1 for CP	Ramp from 47 to 50 kHz	Ramp from 56 to 60 kHz	Ramp from 95 to 100 kHz	Ramp from 80 to 84 kHz
^{13}C ν_1 for CP	-	-	83 kHz	78 kHz
^{15}N ν_1 for CP	58 kHz	52 kHz	-	-
Number of CP loops	10	5	-	-
Inter CP delay	2.5 s	6.0 s	-	-
Number of scans	5120	1536	1024 (μw-on) 14336 (μw-off)	128 (μw-on) 12288 (μw-off)
Acquisition time	10 ms	10 ms	20 ms	20 ms

Additional figures

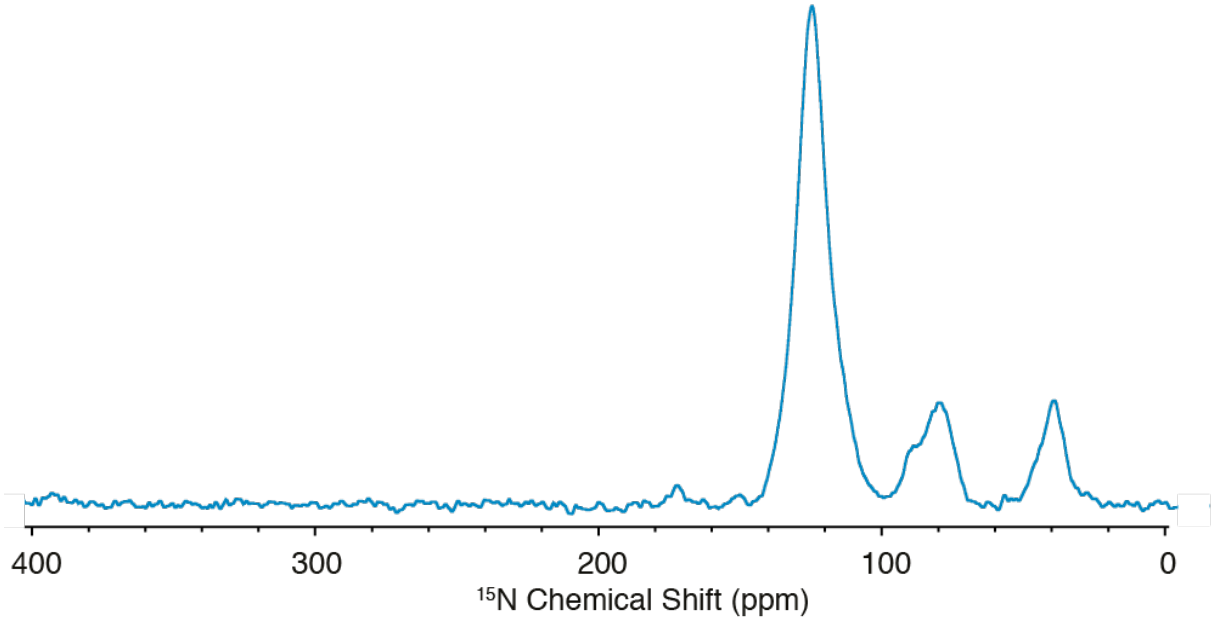


Figure S1. (^{1}H)-^{15}N CP DNP-enhanced spectrum of HEK cells in 14 mM AMUpol, d_6-DMSO:D_2O:H_2O 60:30: $10_{v/v}$ at 11 kHz MAS and ~100 K on a 9.4 T (400 MHz) spectrometer. Spectrum acquired over ~3 days. The region around 370 ppm, where the drug is expected, clearly does not show any signal from the cellular background. The integrals of the intensities corresponding to resonances of the ^{15}N enriched histidine standard (43 ppm; 172 ppm; 250 ppm) are used as a lower limit to quantify background intensities displayed in parenthesis in Figure 2 of the manuscript.

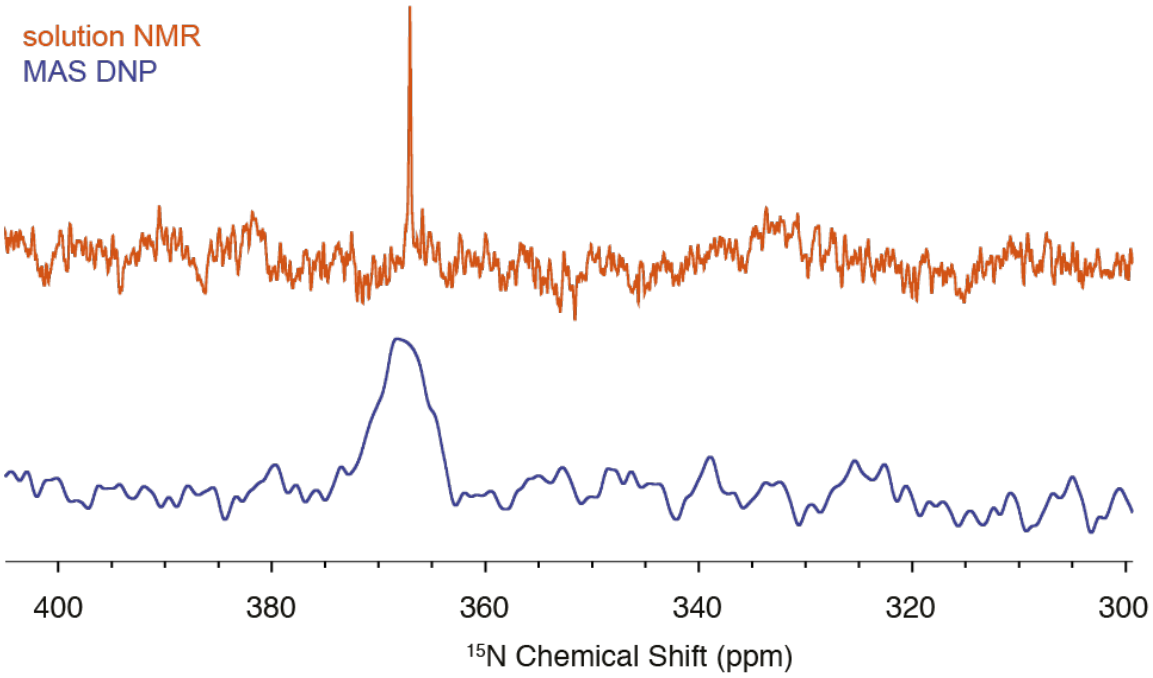


Figure S2. Spectra of pure CHIR-98014. Upper panel (orange): ^{15}N spectrum acquired at 14.1 T (600 MHz) in d_6-DMSO. Lower panel (blue): (^{1}H)-^{15}N CP DNP-enhanced spectrum acquired in 10 mM AMUpol, d_6-DMSO:D_2O:H_2O 60:30: $10_{v/v}$ solution at 8 kHz MAS and ~100 K on a 9.4 T (400 MHz) spectrometer.

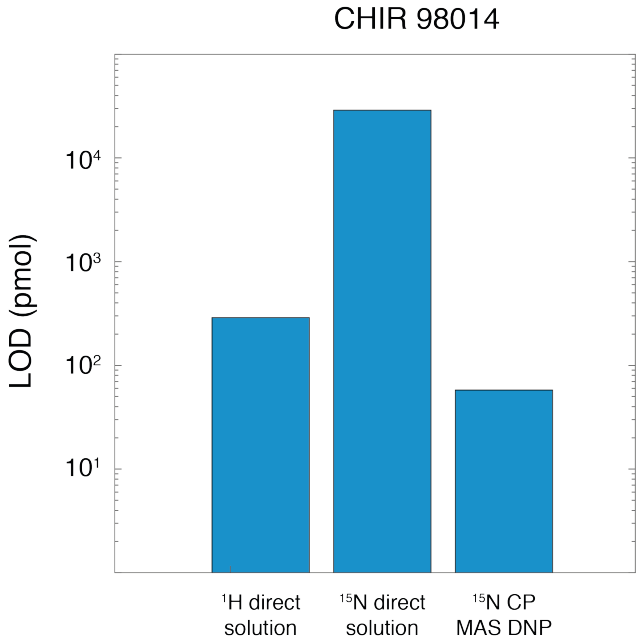


Figure S3. Limit of detection (LOD) of CHIR-98014 in d_6-DMSO (solution NMR) or in 10 mM AMUpol, d_6-DMSO:D_2O:H_2O 60:30: $10_{v/v}$ (MAS DNP), defined as the lowest detectable concentration with a signal-to-noise above ~2 in 72 hours.

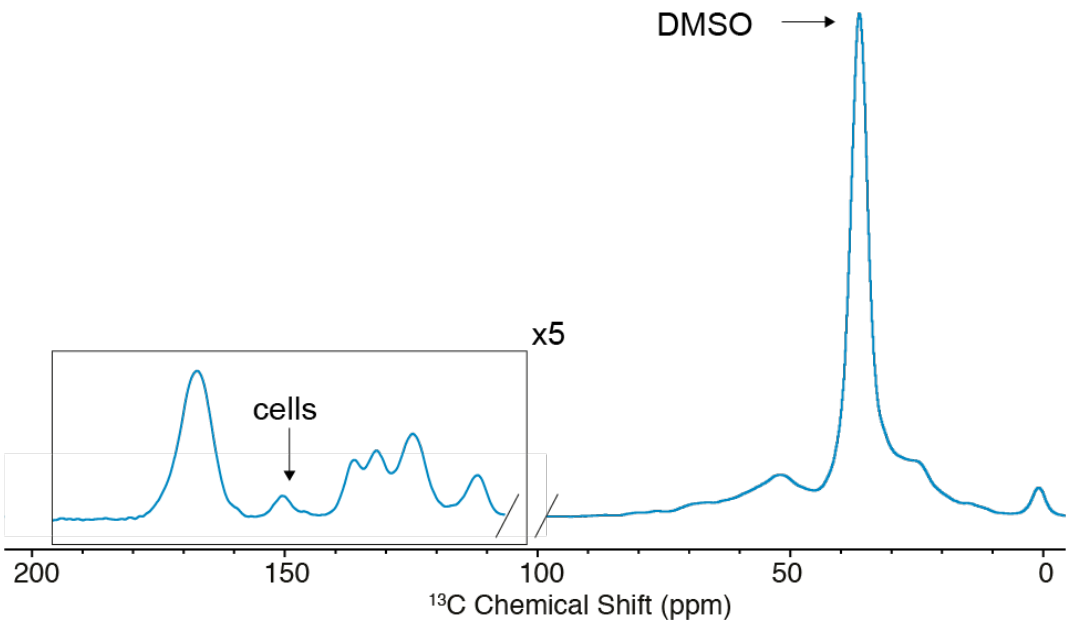


Figure S4. (^{1}H)-^{13}C DNP spectrum of HEK293 cells incubated with 5 μM CHIR-98014 for six hours resuspended in d_6-DMSO:D_2O:H_2O 60:30:10$_{v/v}$, AMUpol 14 mM and ^{15}N, ^{13}C L-histidine·HCl 343 μM. Spectrum acquired at 10.5 kHz MAS and 100 K on a 9.4 T (400 MHz) spectrometer. The peaks used to estimate the enhancements of the cells and of the solvent, DMSO, are indicated by the arrows.

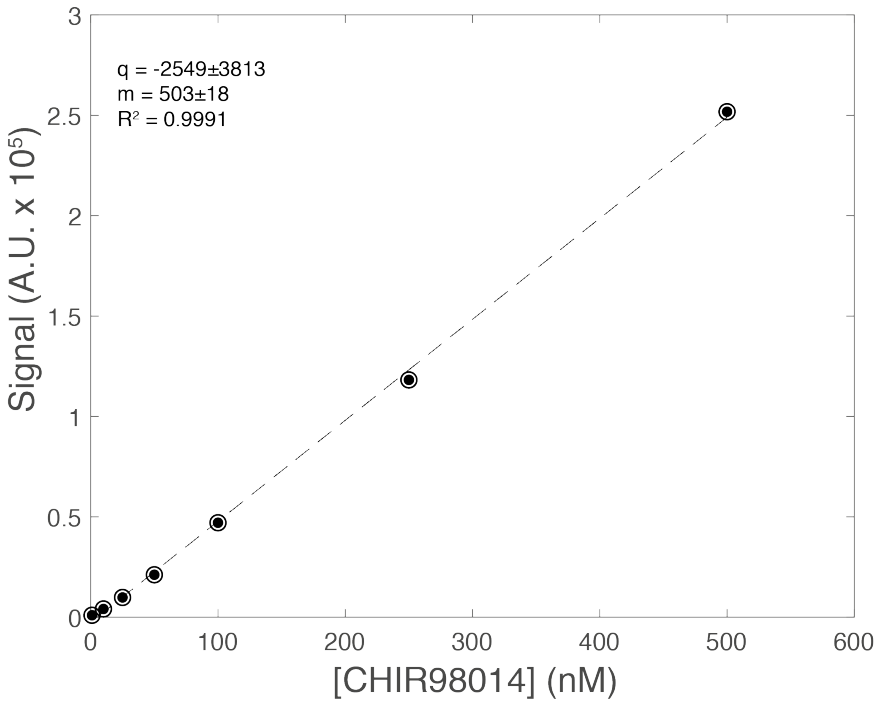


Figure S5. LC-MS calibration curve for the quantification of CHIR-98014. q and m indicate the intercept and the slope, respectively, of the linear fitting of the signal intensity as a function of CHIR-98104 concentration.

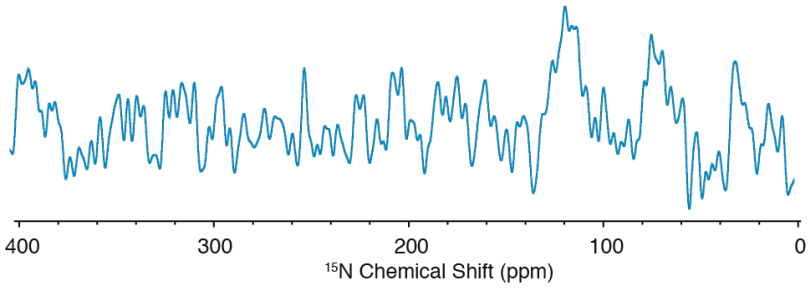


Figure S6. (^{1}H)-^{15}N CP DNP enhanced spectrum of the polarizing solution of HEK293 cells incubated with 10 μM CHIR-98014 for six hours resuspended in d_6-DMSO:D_2O:H_2O 60:30:10$_{v/v}$, AMUpol 14 mM. Spectrum acquired at 10 kHz MAS and 100 K on a 9.4 T (400 MHz) spectrometer for 12 hours.

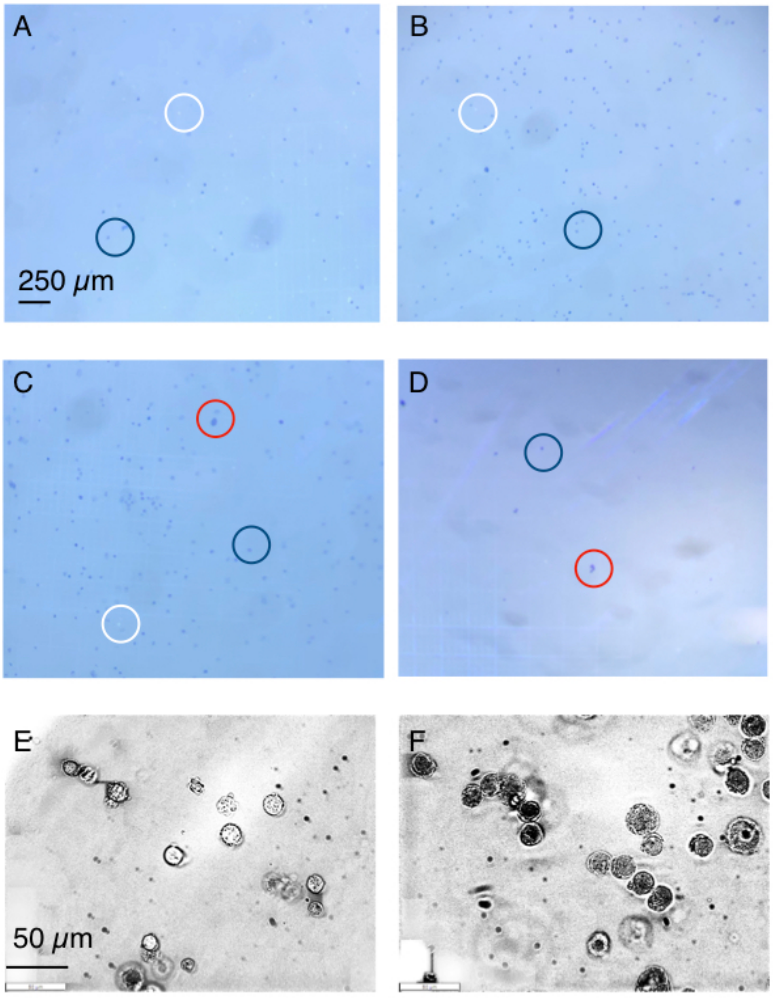


Figure S7. Trypan blue exclusion assays of HEK cells ($3{\cdot}10^6\,mL^{-1}$), and divided in four identical aliquots (as described in the experimental). In this assay cells that are viable exclude the dye and appear as white dots (examples highlighted by white circles) and cells that remain intact but are not viable take up the dye and appear as blue dots (examples highlighted by blue circles). Red circles highlight aggregates. (A) Cells pelleted and resuspended in 15% DMSO, 75% D_2O, 10% H_2O, show viability of ~70%. (B) same as (A), with the addition of a freeze-thaw cycle in the spectrometer, with cells showing viability of <10%. (C) cells resuspended in 60% DMSO, 30% D_2O, 10% H_2O and frozen directly in the spectrometer show viability < 10 %. In (A), (B) and (C) the cell density ($\sim 2.0{\cdot}10^6\,mL^{-1}$) is compatible with the initial cell density ($3{\cdot}10^6\,mL^{-1}$), suggesting that no extensive lyses has occurred. (D) For comparison, cells completely lysed by treatment with acetonitrile followed by a freeze-thaw cycle are no longer observable. (E) and (F) Same as (A) and (C) with higher magnifying power.

References

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