Supplementary data

Concise synthesis and biological activity evaluation of novel pyrazinyl-aryl urea derivatives against several cancer cell lines which especially can induce T24 apoptotic and necroptotic cell death

Jia-Nian Chen[1,*], Chu-Ting Chen[1], Yue-Zhen He[1], Tai-Sheng Qin[1], Li Cheng[1], Ye-Xiang Sun, Kang-Jian Yang, Qi Chen, Chao Yang and Ying Wei

State Key Laboratory for Chemistry and Molecular Engineering of Medicinal Resources, Collaborative Innovation Center for Guangxi Ethnic Medicine, School of Chemistry and Pharmaceutical Sciences, Guangxi Normal University, Yucai Road 15, Guilin 541004, Guangxi, P.R. China

*Corresponding author.

[1]These authors contributed equally to this work.

Correspondence: Jia-Nian Chen

State Key Laboratory for Chemistry and Molecular Engineering of Medicinal Resources, Collaborative Innovation Center for Guangxi Ethnic Medicine, School of Chemistry and Pharmaceutical Sciences, Guangxi Normal University, Yucai Road 15, Guilin 541004, Guangxi, P.R. China

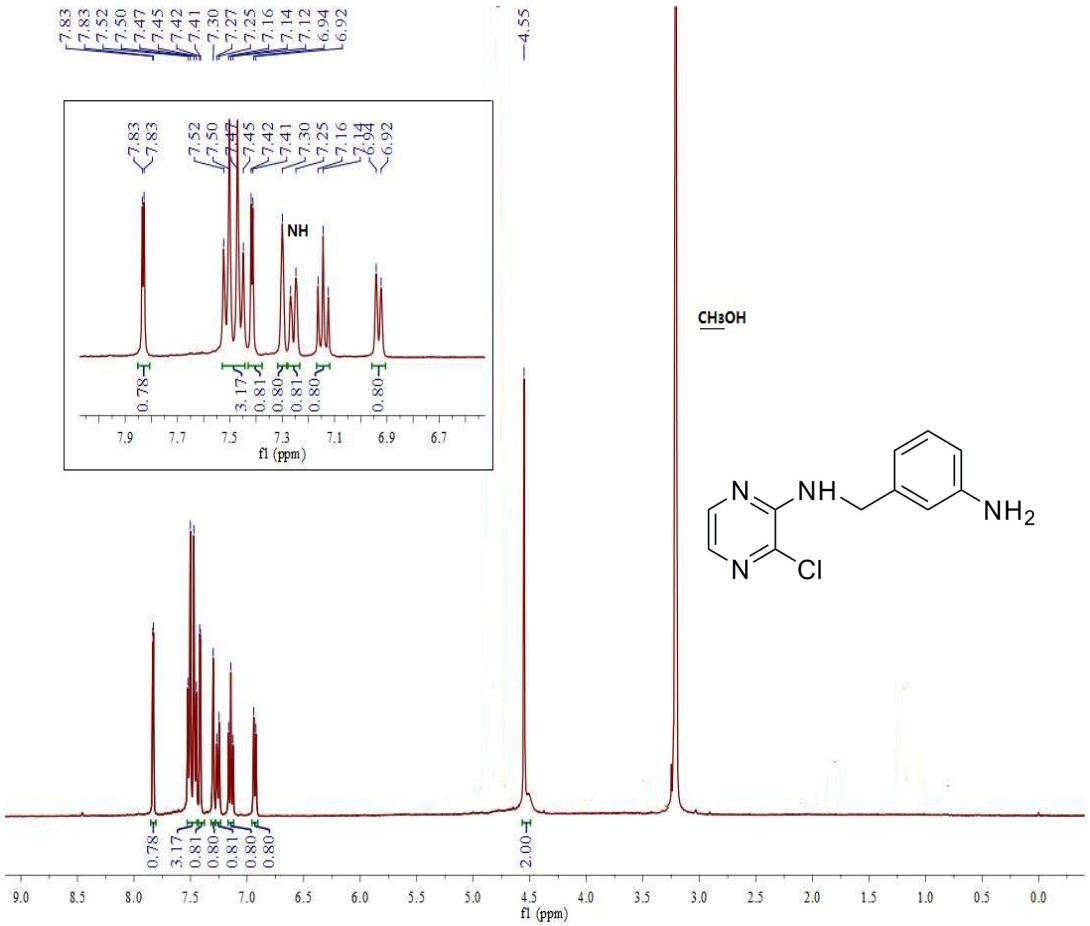
Tel/Fax: +86-0773-2120958

Email: cjn288@mailbox.gxnu.edu.cn

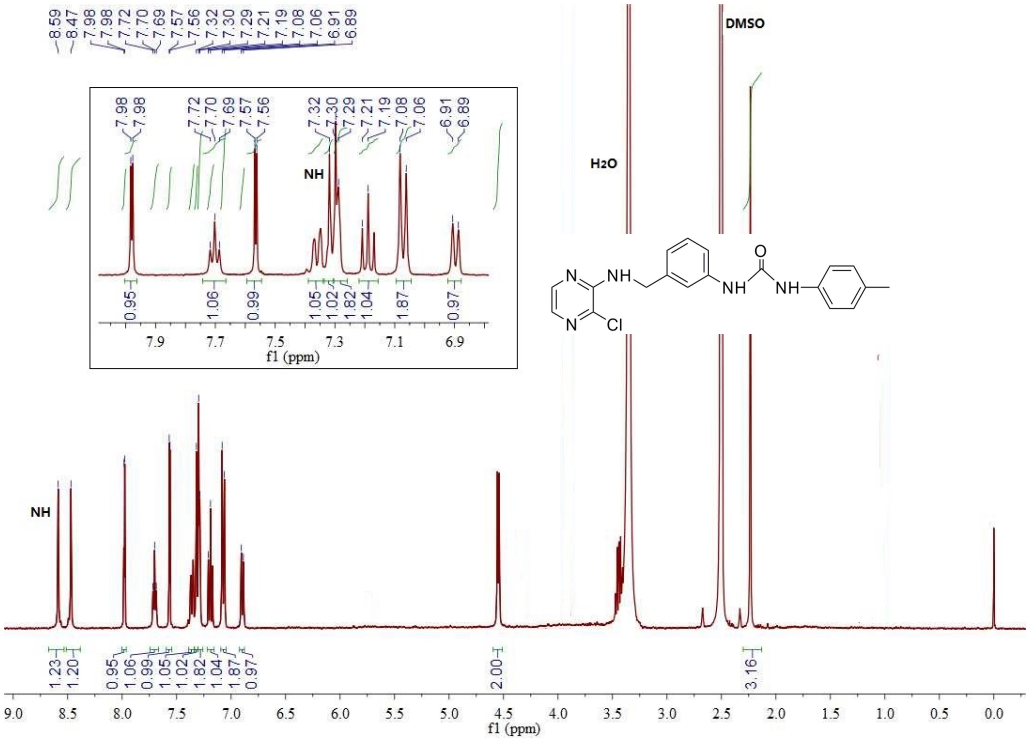
ORCID: 0000-0002-5551-8240

Additional file 1: Spectra of representative compounds and *in vivo* pharmacokinetic study

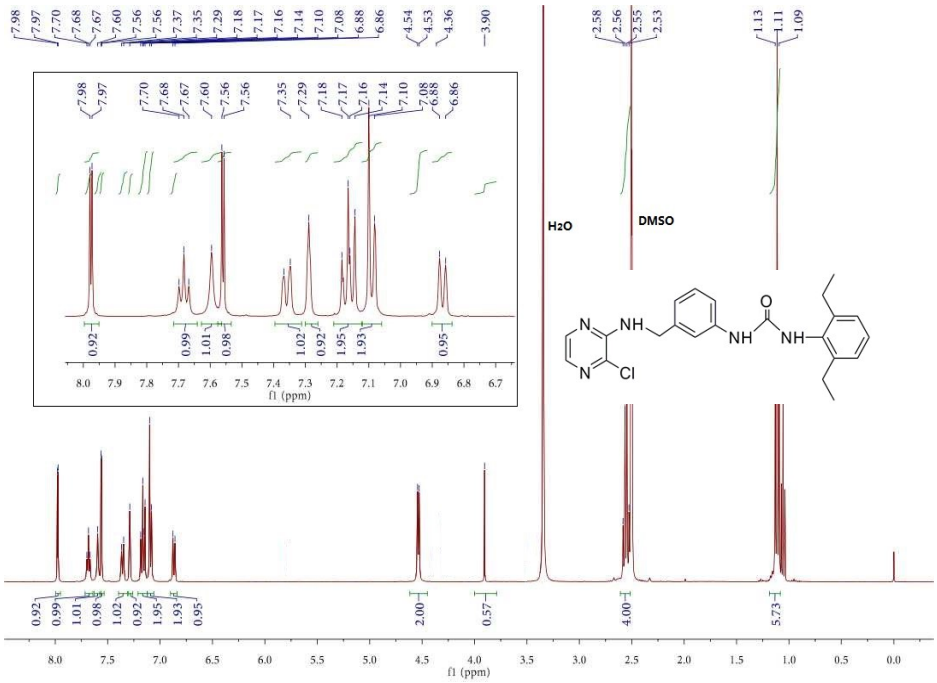
1. ^{1}H NMR spectra



N-(3-Aminobenzyl)-3-chloropyrazin-2-amine (**2**)



N-(4-Methylphenyl)-*N*′-(3-(((3-chloropyrazin-2-yl)amino)methyl)phenyl)urea (**5-4**)



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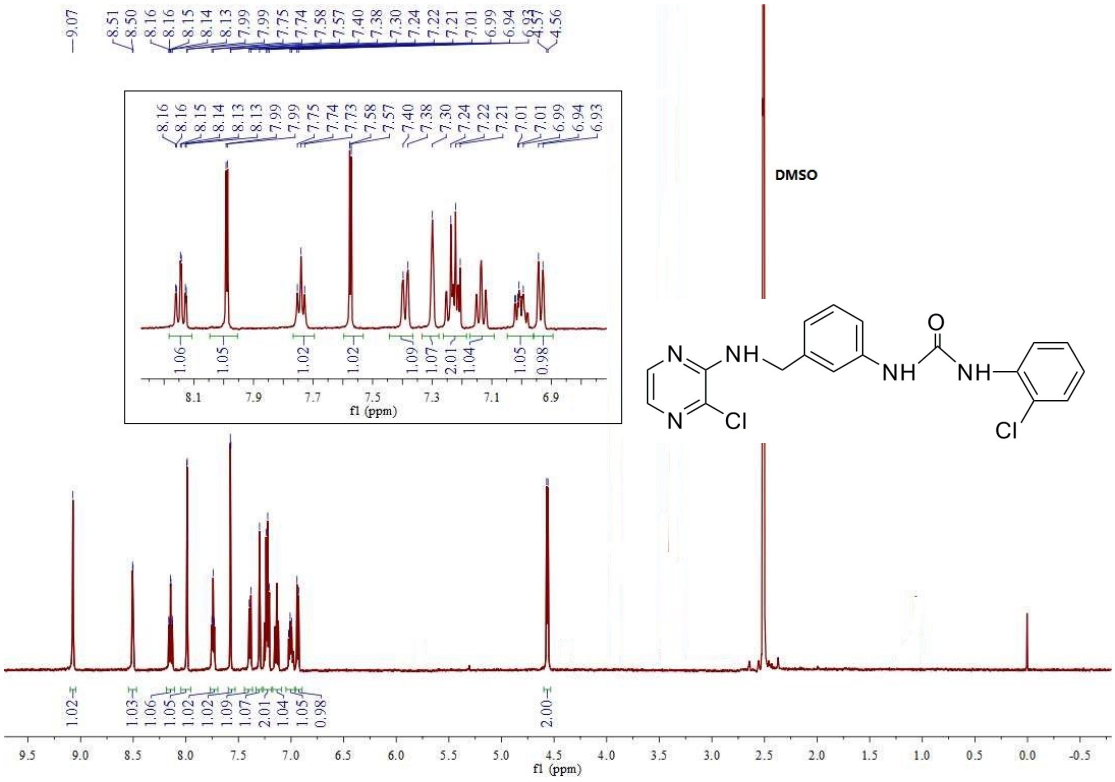
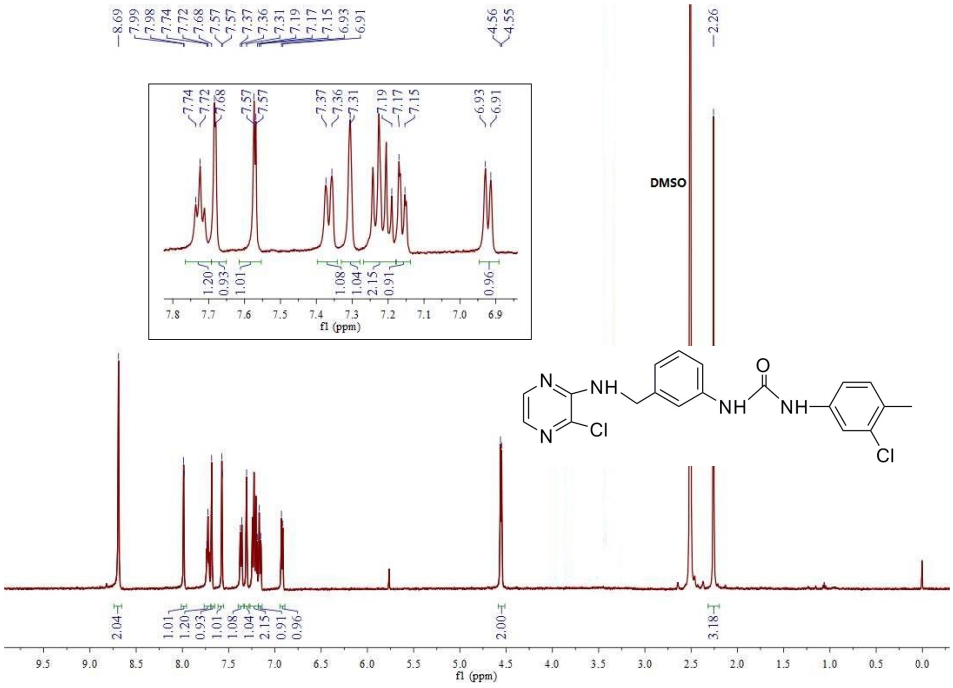
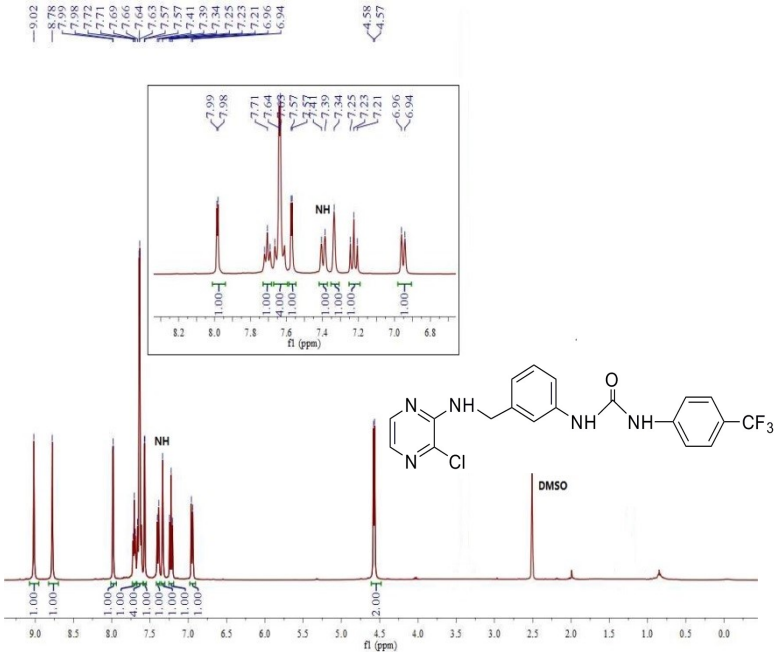


N-(2-Chlorophenyl)-*N*′-(3-(((3-chloropyrazin-2-yl)amino)methyl)phenyl)urea (**5-14**)



N-((3-Chloro-4-methyl)phenyl)-*N*′-(3-(((3-chloropyrazin-2-yl)amino)methyl)phenyl)-urea (**5-20**)



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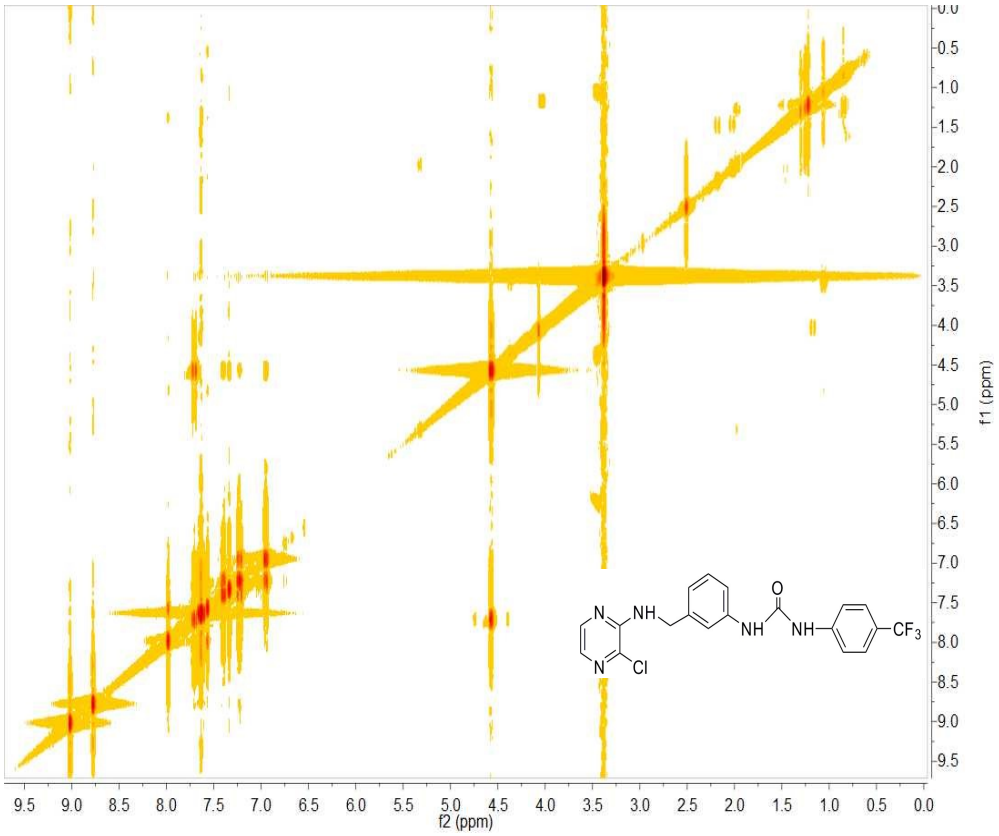
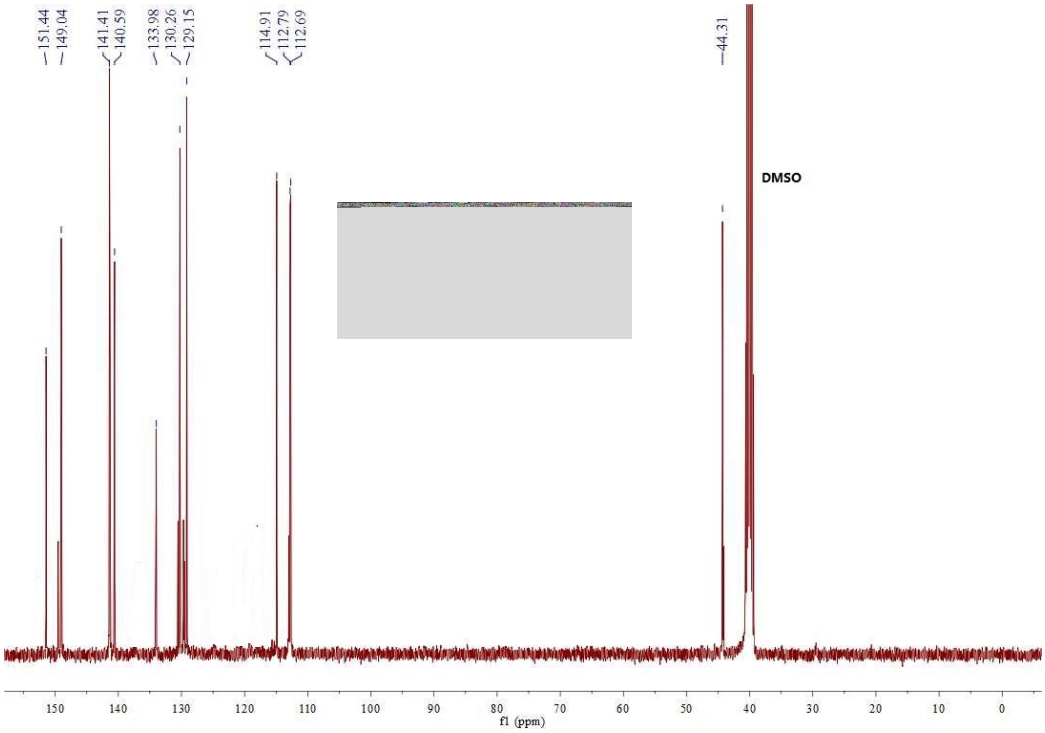
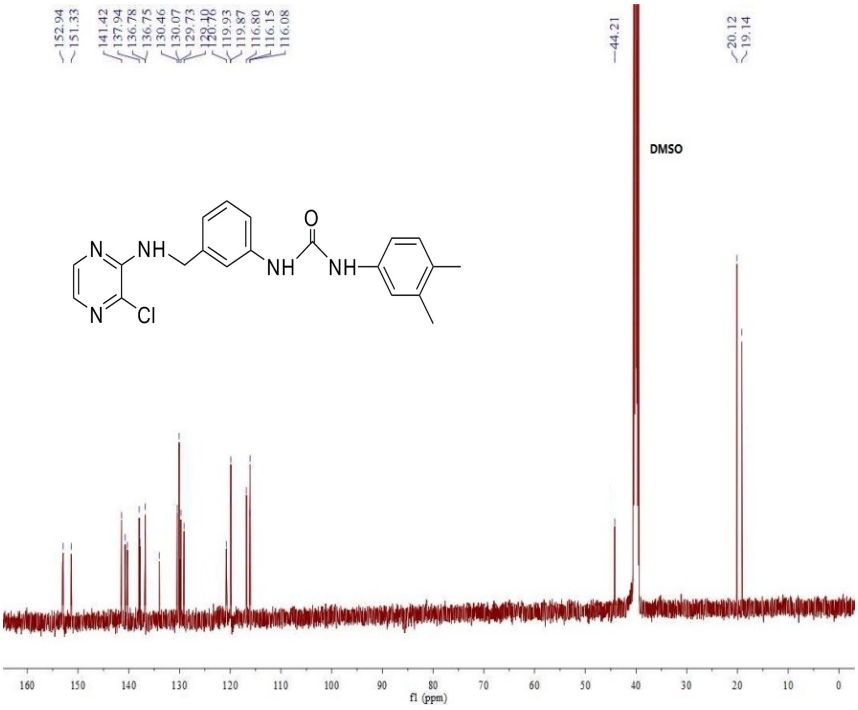


2D-NMR: *N*-((4-Trifluoromethyl)phenyl)-*N*′-(3-(((3-chloropyrazin-2-yl)amino)-methyl)phenyl)urea (**5-23**)

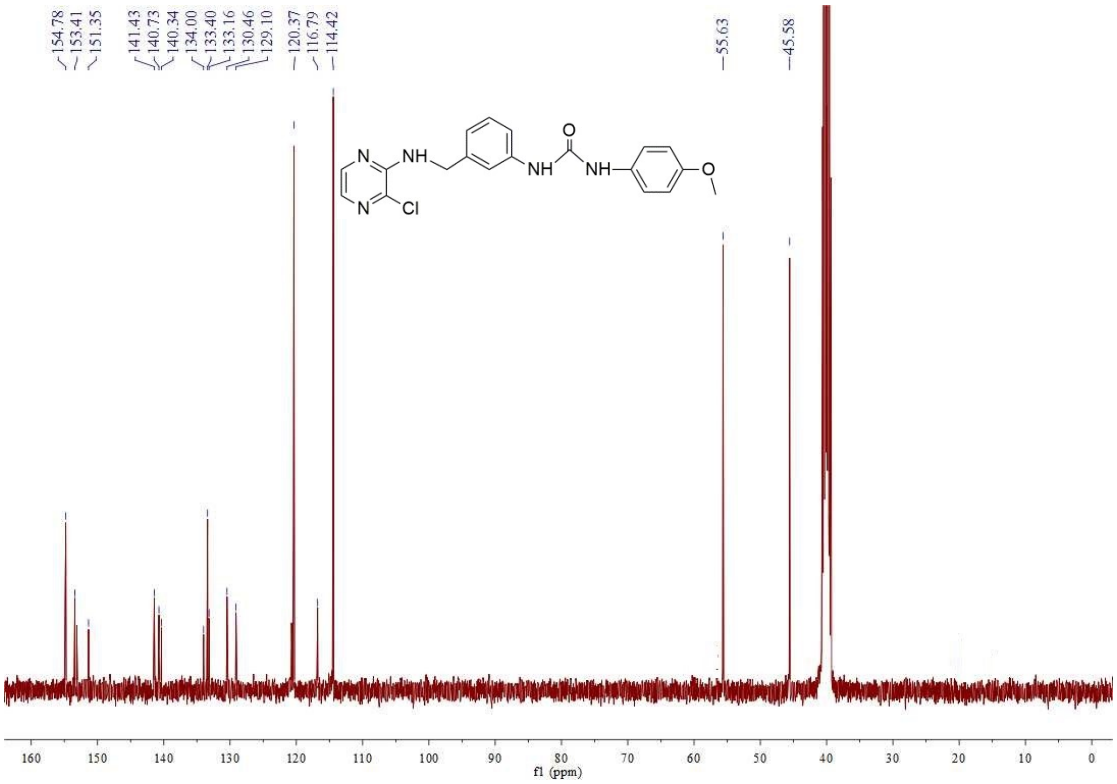
2. ^{13}NMR NMR spectra



N-(3-Aminobenzyl)-3-chloropyrazin-2-amine (**2**)



N-(3,4-Dimethylphenyl)-*N′*-(3-(((3-chloropyrazin-2-yl)amino)methyl)phenyl)urea (**5-6**)



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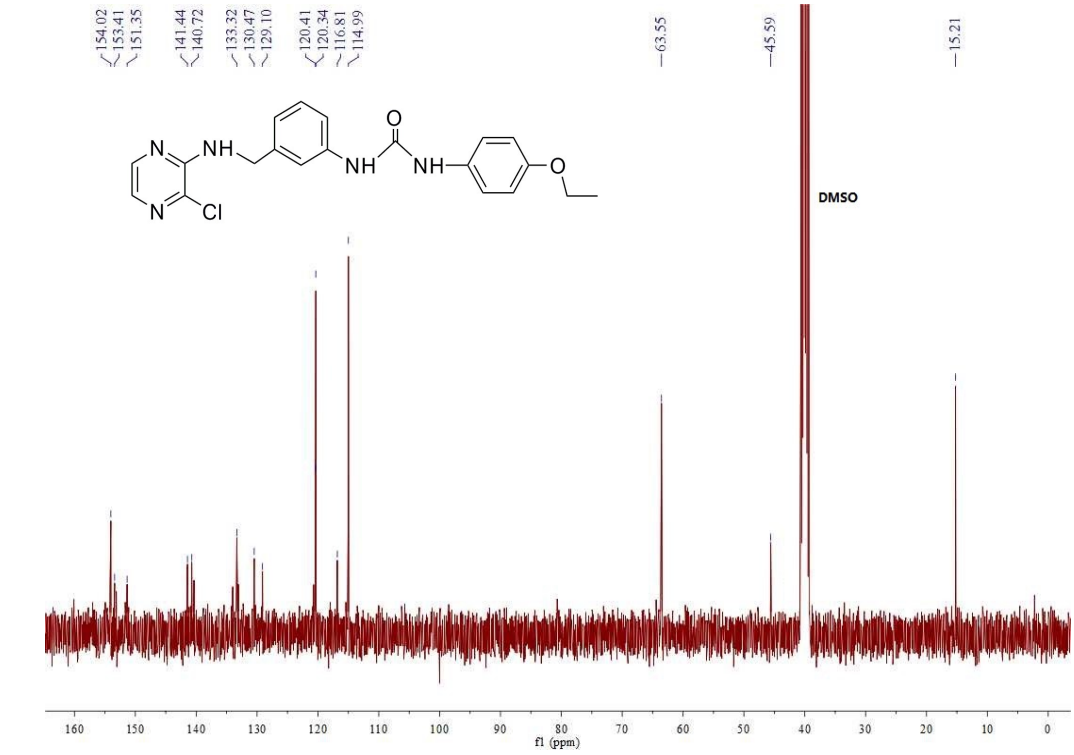

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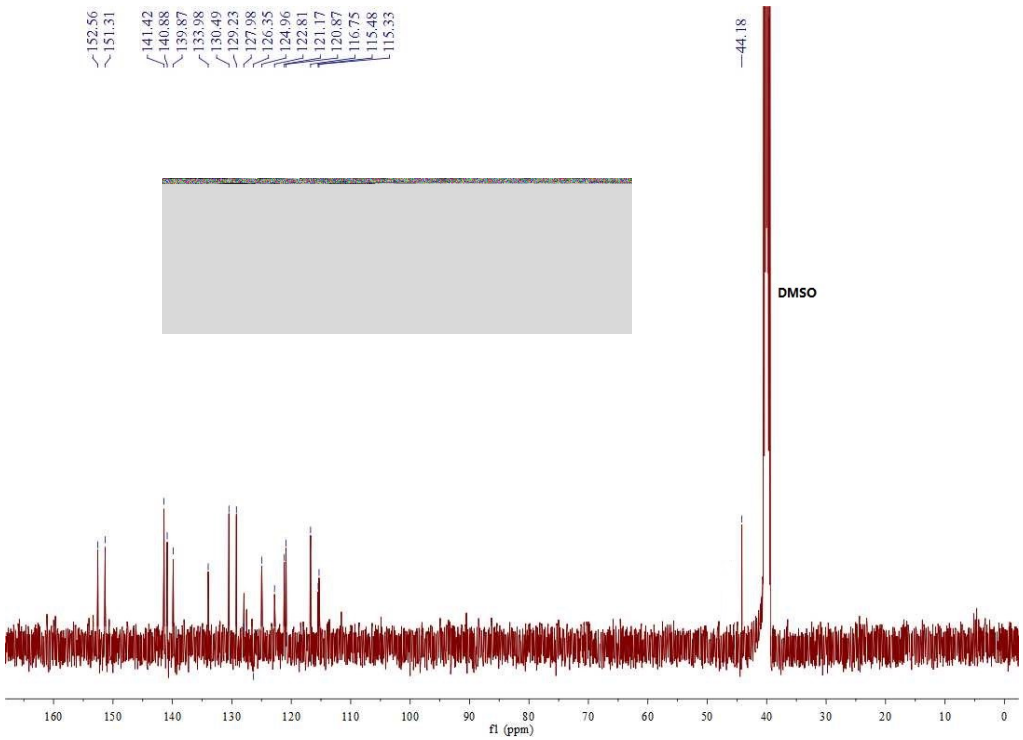
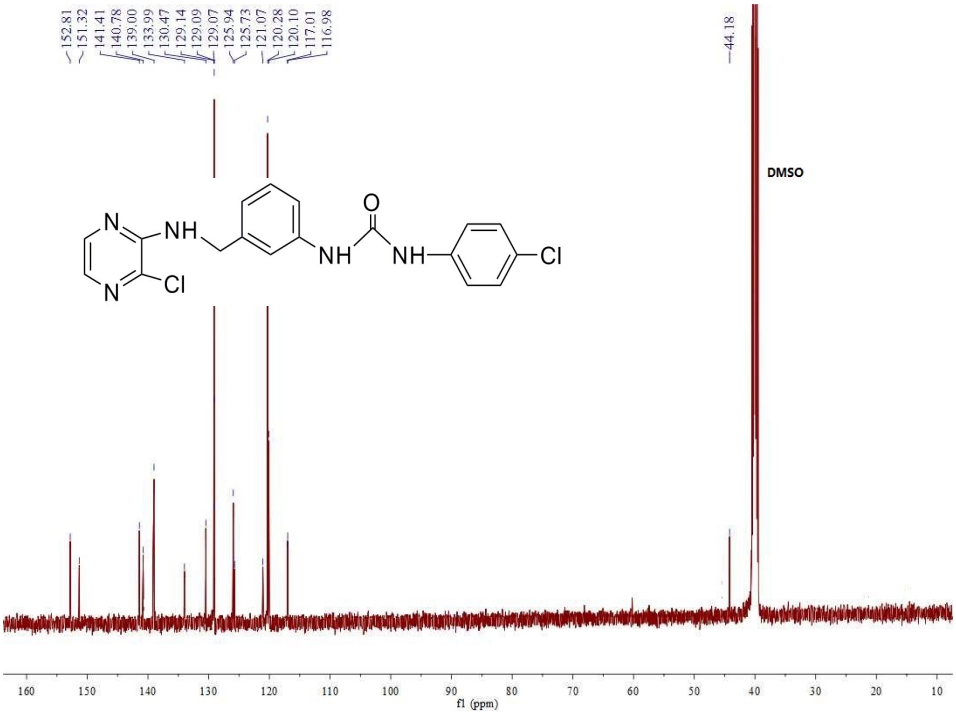
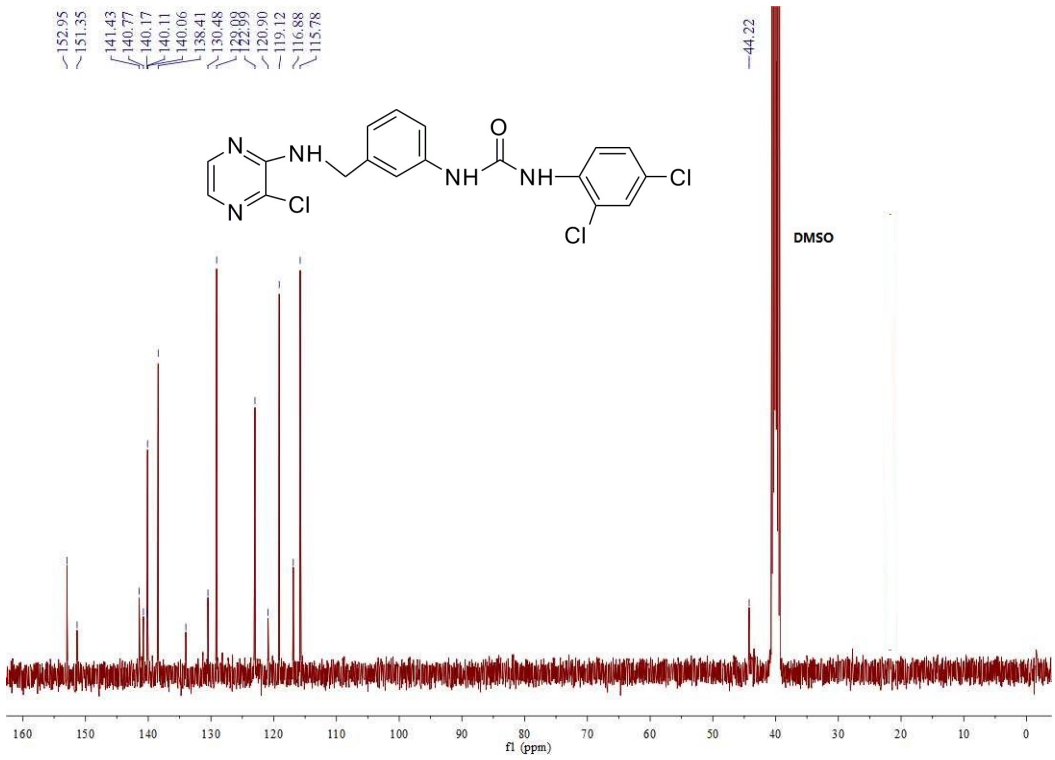


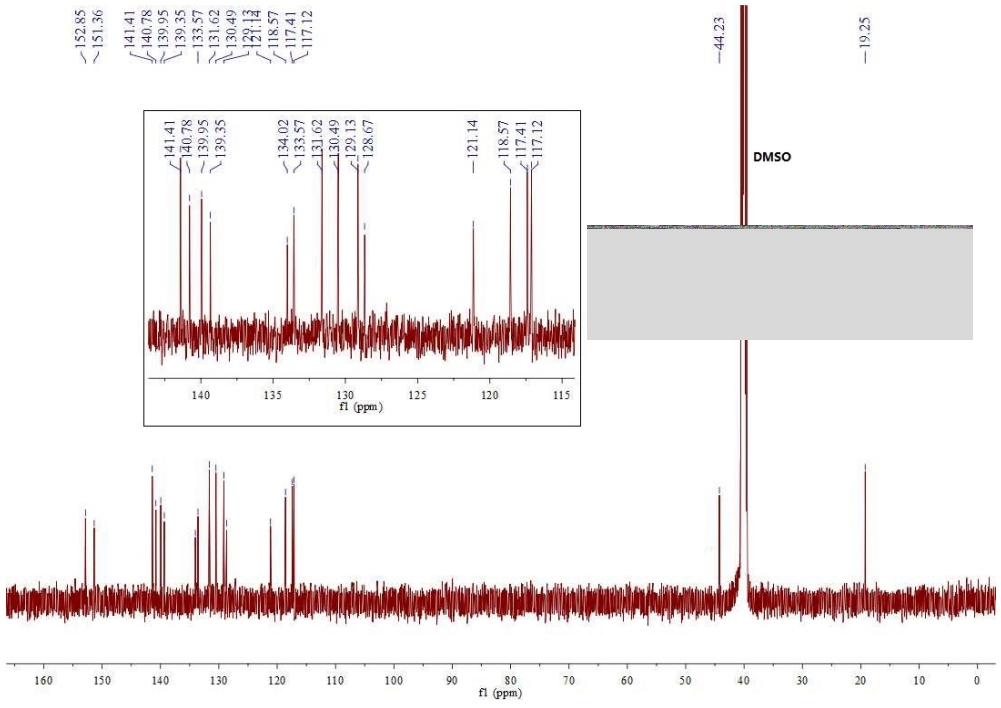
N-(2-Chlorophenyl)-*N′*-(3-(((3-chloropyrazin-2-yl)amino)methyl)phenyl)urea (**5-14**)



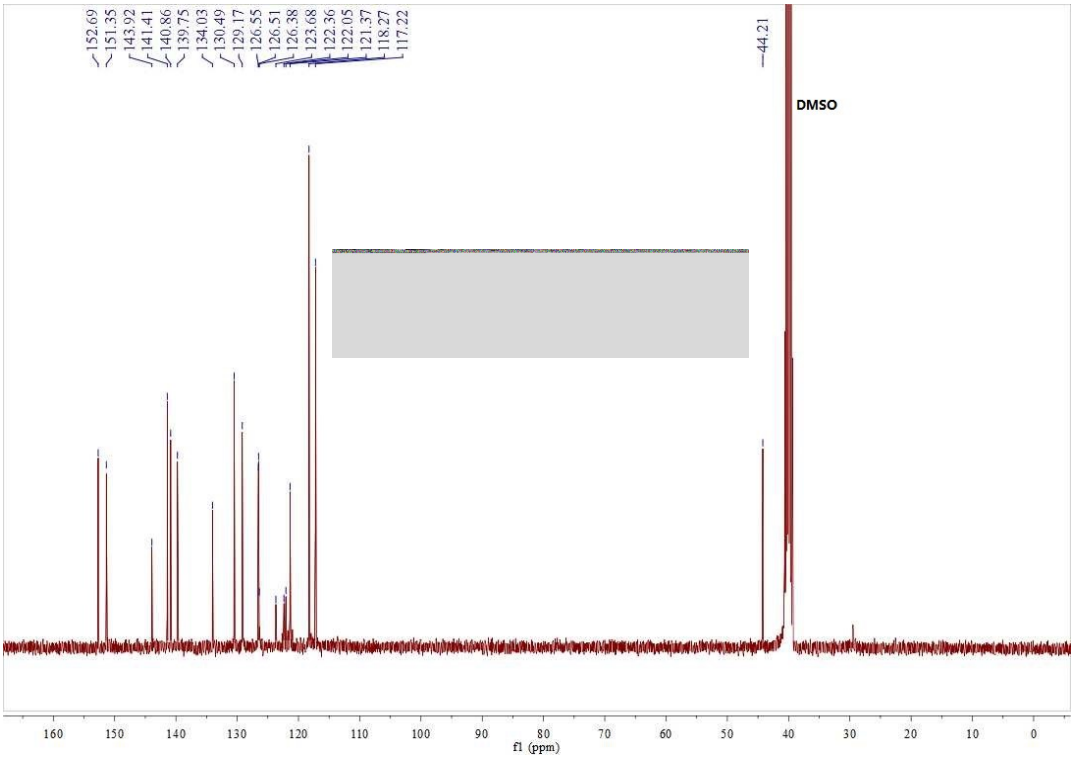
N-(4-Chlorophenyl)-*N′*-(3-(((3-chloropyrazin-2-yl)amino)methyl)phenyl)urea (**5-16**)



N-(2,4-Dichlorophenyl)-*N′*-(3-(((3-chloropyrazin-2-yl)amino)methyl)phenyl)urea (**5-18**)

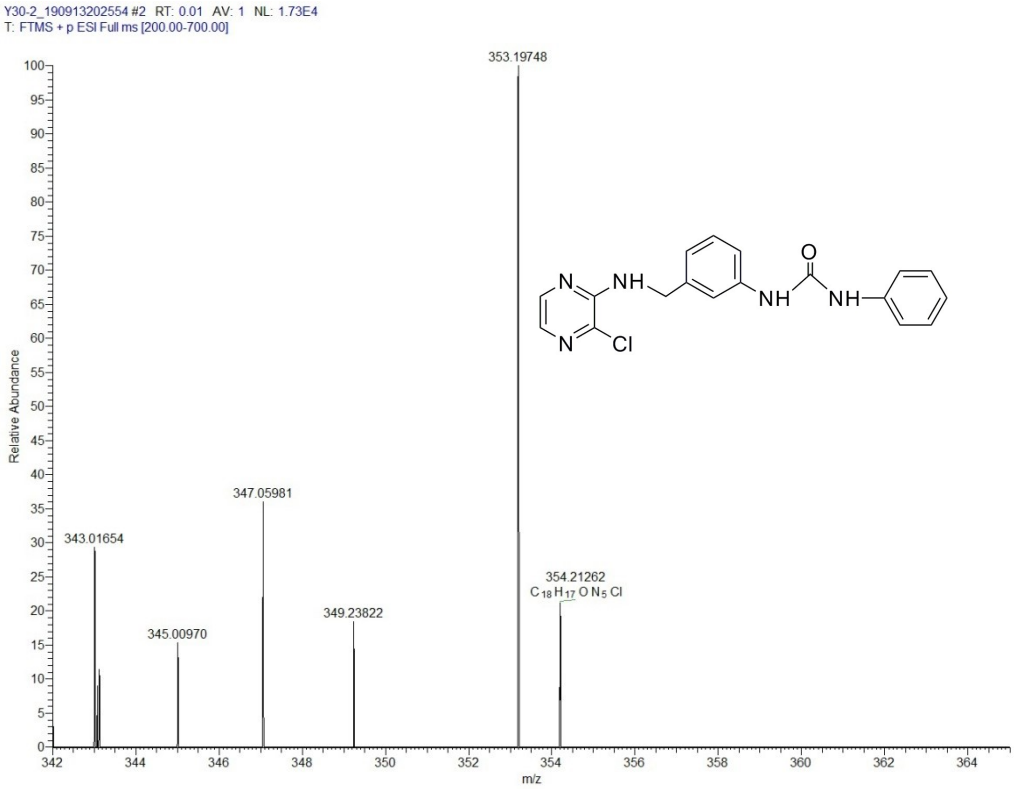


N-((3-Chloro-4-methyl)phenyl)-*N′*-(3-(((3-chloropyrazin-2-yl)amino)methyl)phenyl)-urea (**5-20**)

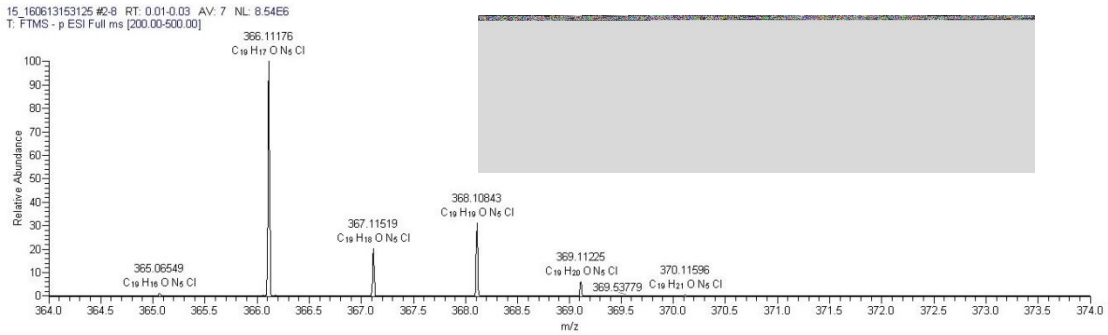


N-((4-Trifluoromethyl)phenyl)-*N*′-(3-(((3-chloropyrazin-2-yl)amino)methyl)phenyl)urea (**5-23**)

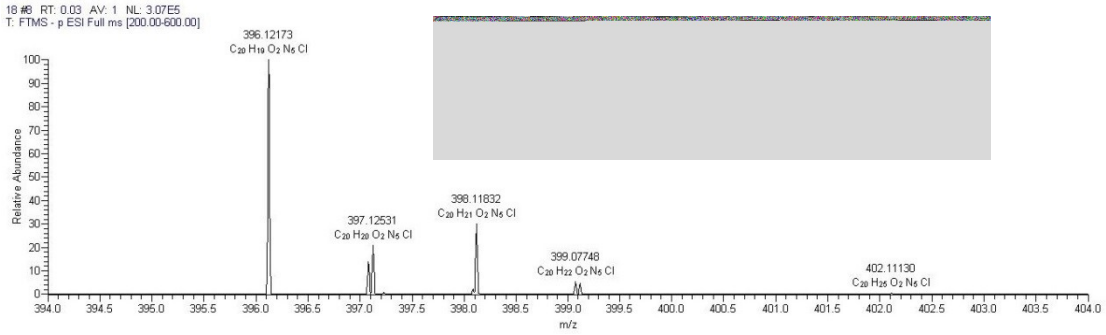
3. HRMS spectra



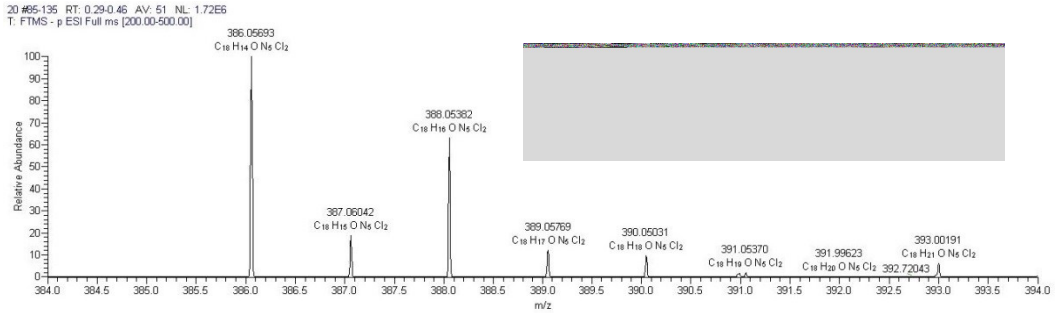
N-Phenyl-*N*′-(3-(((3-chloropyrazin-2-yl)amino)methyl)phenyl)urea **(5-1)**



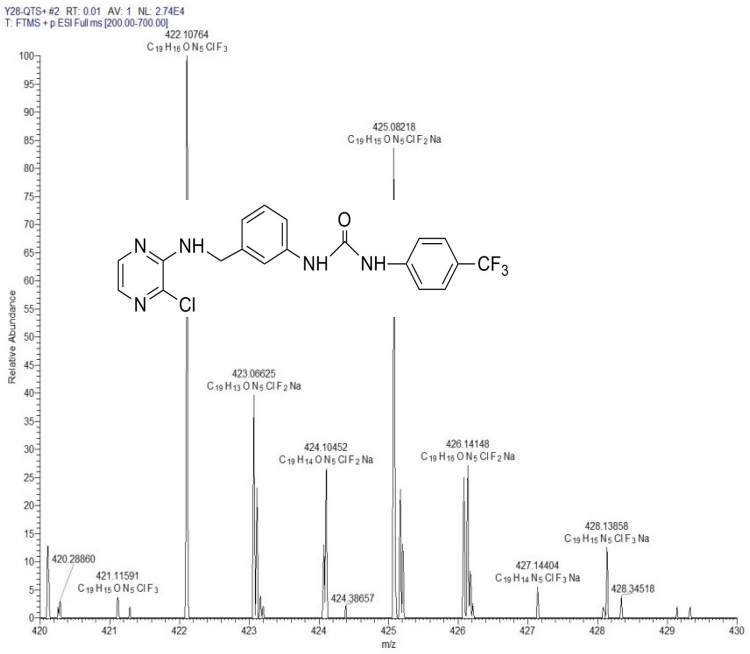
N-(4-Methylphenyl)-*N*′-(3-(((3-chloropyrazin-2-yl)amino)methyl)phenyl)urea (**5-4**)



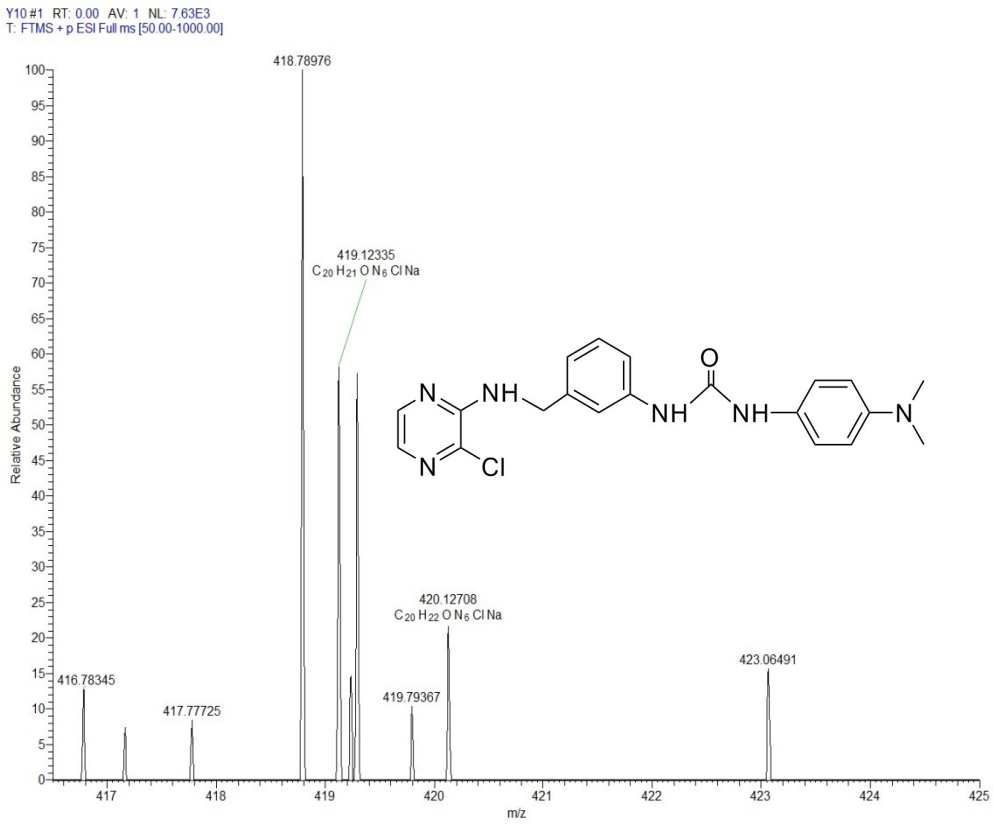
N-(4-Ethoxyphenyl)-*N′*-(3-(((3-chloropyrazin-2-yl)amino)methyl)phenyl)urea (**5-12**)



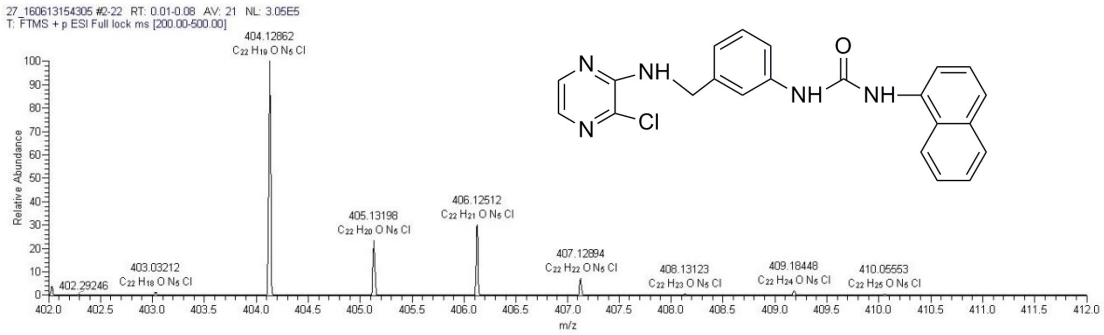
N-(4-Chlorophenyl)-*N′*-(3-(((3-chloropyrazin-2-yl)amino)methyl)phenyl)urea (**5-16**)



N-((4-Trifluoromethyl)phenyl)-*N′*-(3-(((3-chloropyrazin-2-yl)amino)methyl)phenyl)urea (**5-23**)



N-(4-(*N*,*N*-Dimethylamino)phenyl)-*N*′-(3-(((3-chloropyrazin-2-yl)amino)methyl)phenyl)urea (**5-24**)



N-(α-Naphthyl)-*N*′-(3-(((3-chloropyrazin-2-yl)amino)methyl)phenyl)urea (**5-26**)

4. *In vivo* pharmacokinetic study

4.1 Methods

4.1.1 Standard solutions and plasma samples

Stock solutions (200 μg/mL) of **5-23** in methanol were prepared and stored at −20 °C. The working solutions of **5-23** with 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 20.0, 40.0, and 60.0 μg/mL were prepared by serial dilution of the stock solution. Drug-free rat plasma containing sodium heparin as the anticoagulant was obtained from Sprague-Dawley (SD) rats. Plasma calibration standards of **5-23** (25, 50, 100, 250, 500, 1000, 2000, 4000, and 6000 ng/mL) were prepared by adding 25 μL of working solution to 250 μL drug-free rat plasma. Plasma calibration standards of Reg were prepared according to the same procedures mentioned above, and the following procedures were described in Section 4.1.2.

Quality control (QC) samples were separately prepared in a similar way as calibration standards, and the concentrations of 100, 500, and 4000 ng/mL of **5-23** in plasma samples were corresponding to low, medium, and high QC, respectively. QC samples were analyzed six times for each concentration using HPLC.

4.1.2 Blood sample post-processing

After plasma calibration standards were obtained, 1000 μL of acetonitrile was added to precipitate protein in the blood. After centrifuging at 3000 rpm for 10 min, the supernatant was collected, transferred into a tube, and dried under a stream of nitrogen. The residue was reconstituted in 250 μL of the mobile phase, filtered through a disposable Millipore filter (0.45 μm pore size), and then 20 μL of the sample was injected into the HPLC for analysis.

4.2 Method validation

4.2.1 Calibration curves

Every calibration standard concentration was assayed six times. After injecting all the processed calibration standard samples of various concentrations covering the

working range of the assay, the calibration curves were established in the range of 25–6000 ng/mL. The peak areas of **5-23** were recorded; peak areas and the concentrations of **5-23** were used to plot the calibration curve ($y = a \times x + b$). For Reg, the same method was used. In the above equation, y represents the peak area of **5-23** (or Reg) and x represents the concentration of **5-23** (or Reg).

4.2.2 Specificity

The specificity of the method was investigated by comparing the chromatograms of the following plasma samples after they were collected from the SD rats and treated according to the procedures described in Section 4.1.2. There should be no interference from endogenous or exogenous materials observed at the retention time of **5-23**. The plasma samples in each group were obtained from six different SD rats. For Reg, the procedure was the same as mentioned above.

4.2.3 Precision and accuracy

The precision of the assay was assessed by calculating the relative standard deviation (R.S.D.) for each concentration level, and inter-day precision was determined by the analysis of QC samples on three consecutive days. Accuracy was calculated by comparing the average measurements with the nominal values and was expressed in percent.

4.2.4 Recovery

Recoveries of **5-23** were determined by the analysis of the above three **5-23** QC samples. A 25 μL of the standard solution of **5-23** was transferred into 250 μL of blank rat plasma. Then the samples were treated according to the method described in Section 4.1.2 and the peak area ($S_{\mathbf{5\text{-}23}}$) was obtained. For the reference material, the same concentration standard solution in the mobile phase was injected directly into the HPLC system and the peak area ($A_{\mathbf{5\text{-}23}}$) was also gained. The results of ($S_{\mathbf{5\text{-}23}}/A_{\mathbf{5\text{-}23}}$) were defined as the recoveries of the method and expressed in percent. The

experiments were repeated six times for each concentration.

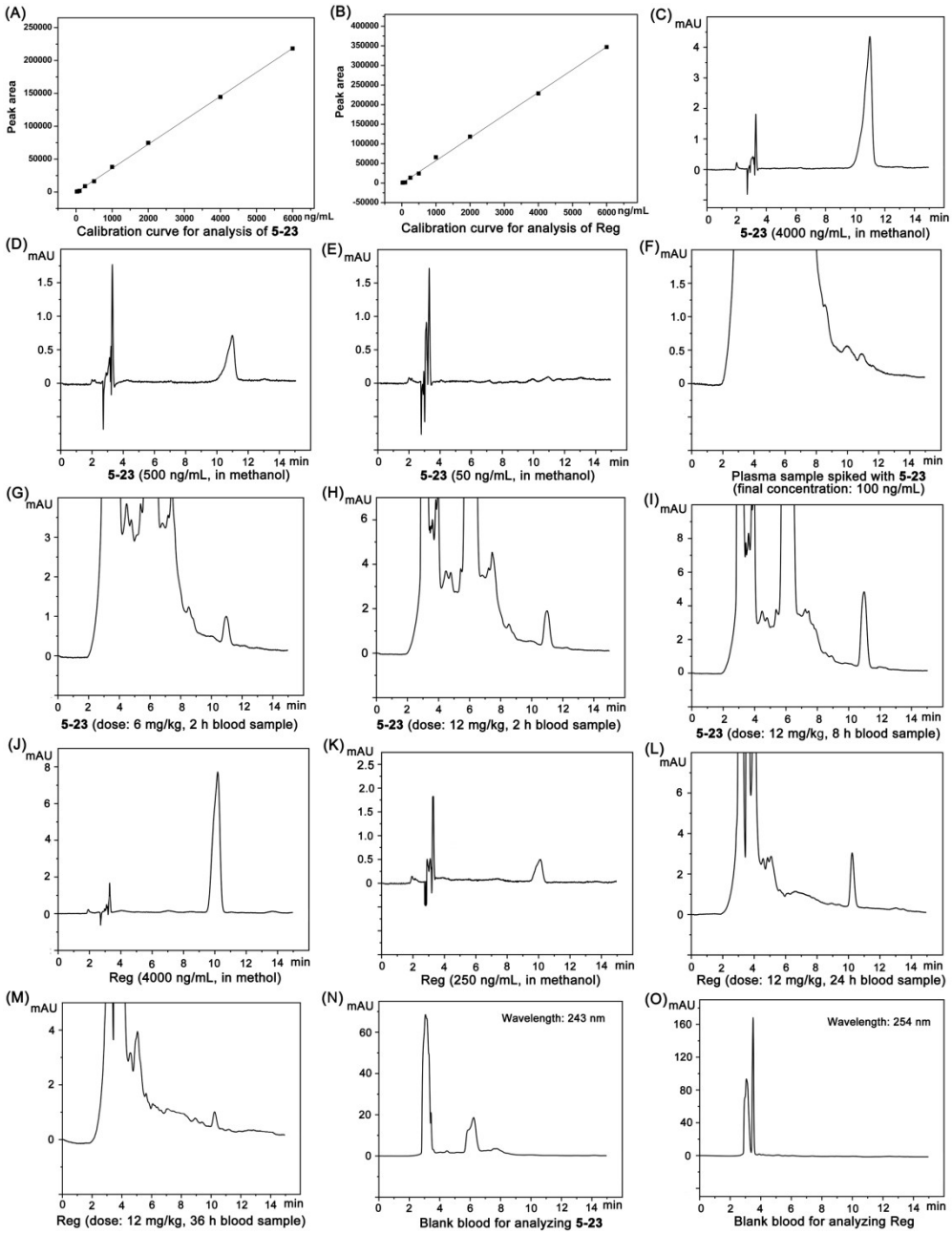


Fig. S4-1 Calibration curves and representative chromatograms of **5-23** and Reg.

4.3 Results and discussion

4.3.1 Suitable HPLC conditions

For regorafenib (Reg), the isocratic mobile phase consisted of 0.02% formic acid in distilled water (eluent A) and methanol (eluent B) with an appropriate volume ratio

(21:79) and flow rate (1.0 ml/min). The column temperature was maintained at 25 °C and the UV-vis detection wavelength was set at 254 nm. The total run time was 15 min. However, the above-mentioned chromatographic conditions were not suitable for analyzing **5-23** because the retention time was too short (< 6 min) if the conditions were adopted. After many attempts, optimized chromatographic conditions for the determination of **5-23** were explored: Mobile phase A consisted of water with 0.1% (v/v) formic acid; mobile phase B consisted of acetonitrile and methanol (1:3) with 0.1% (v/v) formic acid. The gradient elution profile was as follows: 0–2 min (A, 90%; B, 10%), 2–9 min (A, 20%; B, 80%), 9–12 min (A, 8%; B, 92%), 13–15 min (A, 90%; B, 10%). The UV-vis detection wavelength was set at 243 nm. Other settings were the same as those in the Reg analysis.

4.3.2 Calibration curves and linearity

Typical regression equations for **5-23** and Reg were $y = 36.5x - 522.9$ ($r = 0.9976$) and $y = 58.0x - 820.2$ ($r = 0.9959$), respectively. Under the chromatographic conditions mentioned above, the average elution time of **5-23** and Reg was 10.9 min and 10.2 min, respectively. Calibration curves for quantitative analysis of **5-23** and Reg are listed in Fig. S4-1 [see (A) and (B)].

4.3.3 Specificity

Typical HPLC chromatograms and of **5-23** and Reg samples were shown in Fig. S4-1 and they represented the following cases, respectively: (C–E) different concentrations of **5-23** dissolved in methanol and injected into the HPLC system directly; (F) plasma sample spiked with **5-23**; (G–I) plasma sample (different time points) from a rat after intragastric administration of **5-23** at doses of 6 and 12 mg/kg, respectively; (J, K) different concentrations of Reg dissolved in methanol and injected into the HPLC system directly; (L, M) plasma sample (different time points) from a rat after intragastric administration of Reg at doses of 12 mg/kg; (N, O) blank plasma samples detected at 254 and 243 nm, respectively. The blood samples collected were

treated based on the procedures covered in section 4.1.2. Because the conditions for the determination of **5-23** and Reg were different, two blank blood samples were detected during blood sample analysis [see Fig. S4-1 (N) and (O)]. From the results, no interfering peaks were observed at the retention time of **5-23** and Reg, and the assay had adequate specificity.

Table S4-1 The validation of intra- and inter-day precision and accuracy of **5-23** with QC samples (n = 6)

Concentration (ng/mL)	Precision (%R.S.D.)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
100	9.05	10.41	95.29	90.03
500	7.06	7.91	91.05	88.79
4000	4.33	5.72	104.32	95.38

4.3.4 Accuracy and precision

For **5-23** QC samples, intra-day accuracy ranged from 91.05% to 104.32%; and inter-day accuracy ranged from 88.79% to 95.38%. The intra- and inter-day assay precisions (R.S.D.) were ≤ 9.05% and ≤ 10.41%, respectively. Detailed results are presented in Table S4-1.

Table S4-2 Method recoveries of **5-23** from rat plasma (n = 6)

Nominal concentration (ng/mL)	Mean ± S.D. calculated (ng/mL)	Method recoveries (%)[a]
100	86.2 ± 5.9	86.2 ± 6.8
500	446.3 ± 26.4	89.3 ± 5.9
4000	3727.6 ± 108.6	93.2 ± 2.9

[a] Expressed as [(mean calculated concentrations)/(nominal concentrations) × 100].

4.3.5 Recovery

Recoveries of **5-23** were determined by the analysis of the above low, medium, and high QC samples. The recoveries of the method were between 86.2% and 93.2%. Detailed results are presented in Table S4-2.