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

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Additional file 2: Fig. S1–S8 and Table S1–S2

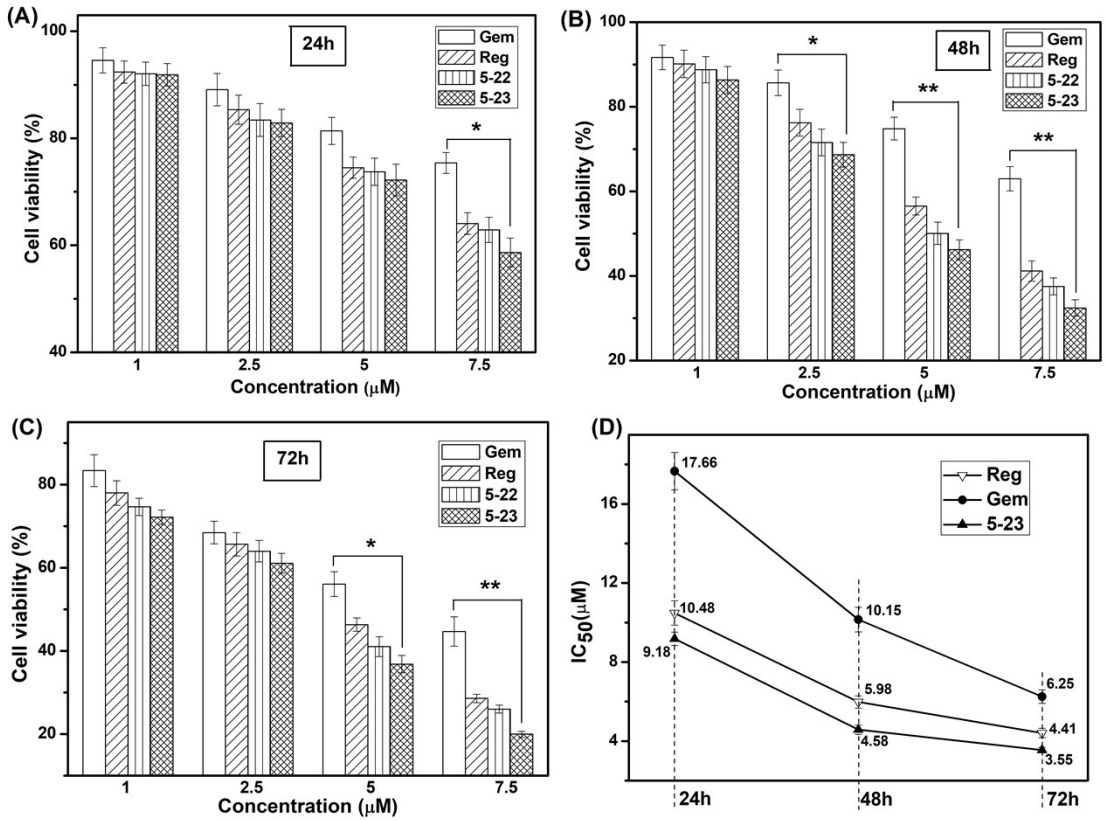


Fig. S1 The inhibitory effects of pyrazinyl-aryl urea derivative **5-23** on T24 cell proliferation. T24 cells were treated with **5-23** (or positive reference drugs regorafenib and gemcitabine) for the indicated periods of time (24, 48, and 72 h), respectively, cell viability was then measured by the MTT assay (A–C). (D) IC_{50} values of **5-23**, Reg, and Gem at different incubation times. Results are presented as means ± S.D. (standard deviation) of four independent experiments. * $p < 0.05$ and ** $p < 0.01$. Abbreviations: Gem, gemcitabine; Reg, regorafenib.

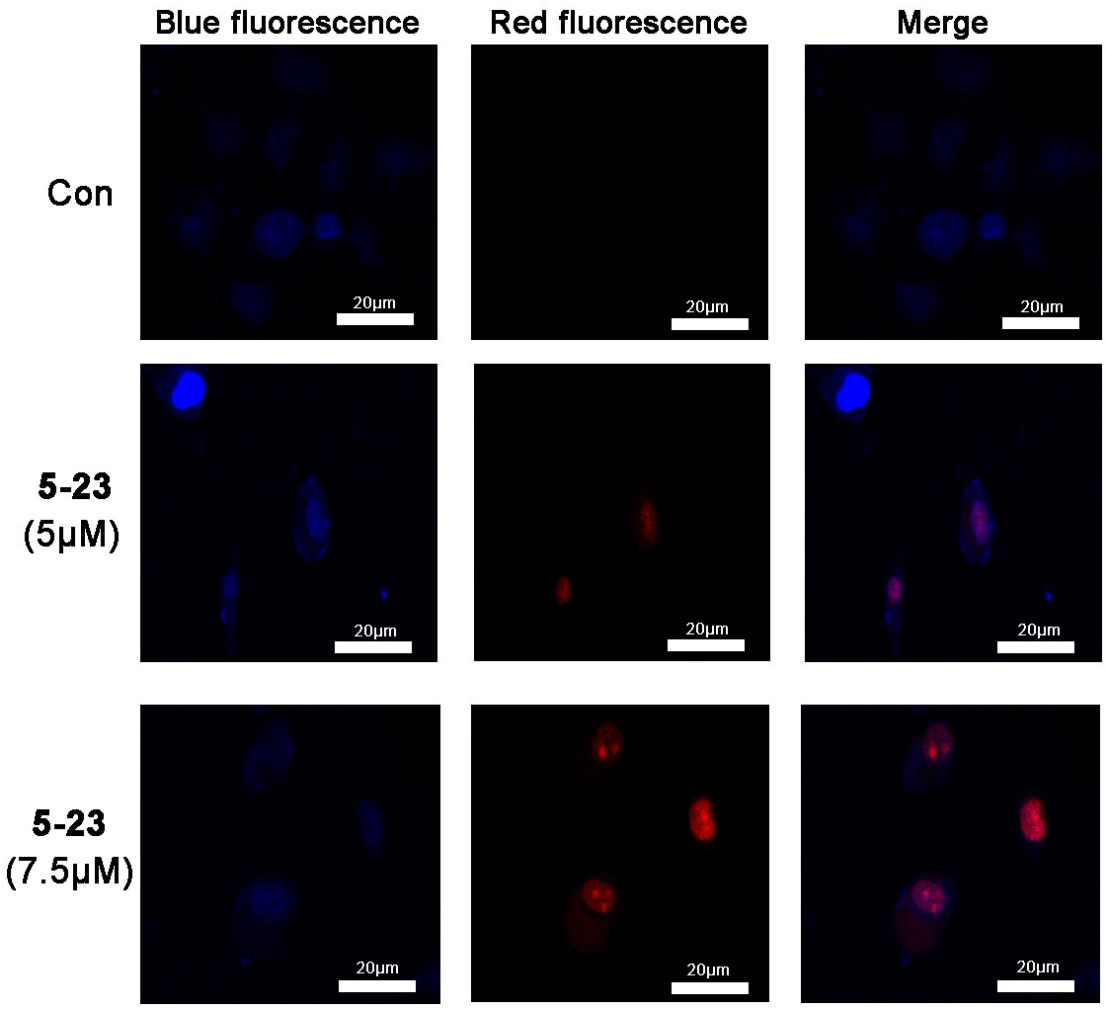


Fig. S2 Induced apoptosis of T24 cells by compound **5-23**. After the co-incubation with **5-23** for 10 h, T24 cells were stained with Hoechst 33342 and propidium iodide (Hoe/PI). The experiment was repeated thrice, here only representative fluorescence microscopic images were shown.

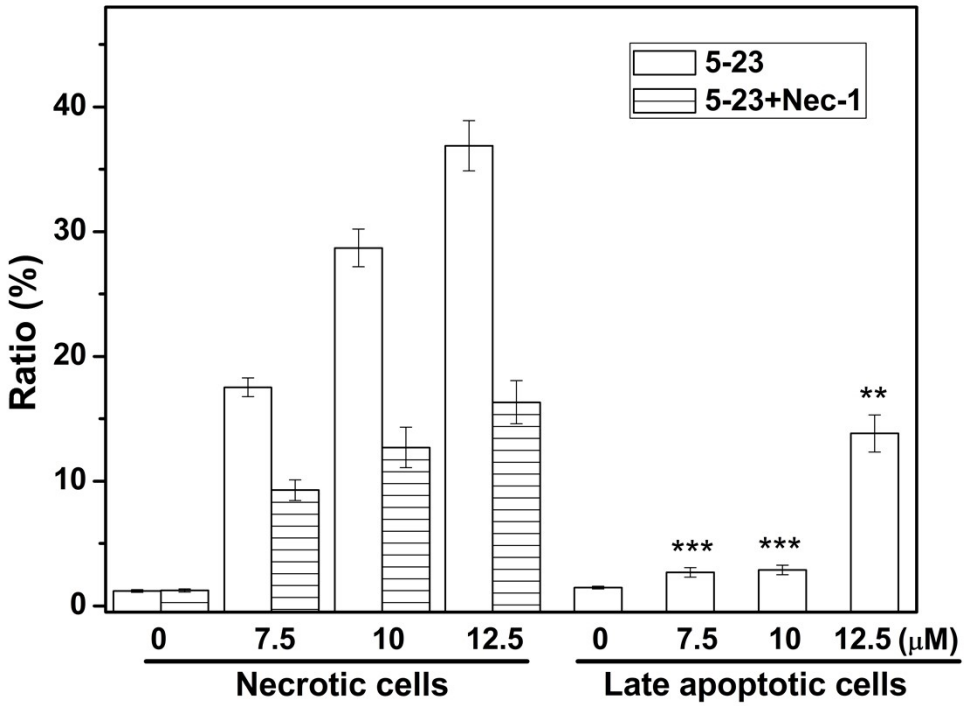


Fig. S3 Changes in the ratios of necrotic and late apoptotic cells after the treatment with **5-23** in the absence or presence of Nec-1. ** $p < 0.01$ and *** $p < 0.001$ versus the ratios of necrotic cells at the same concentration of **5-23** (absence of Nec-1).

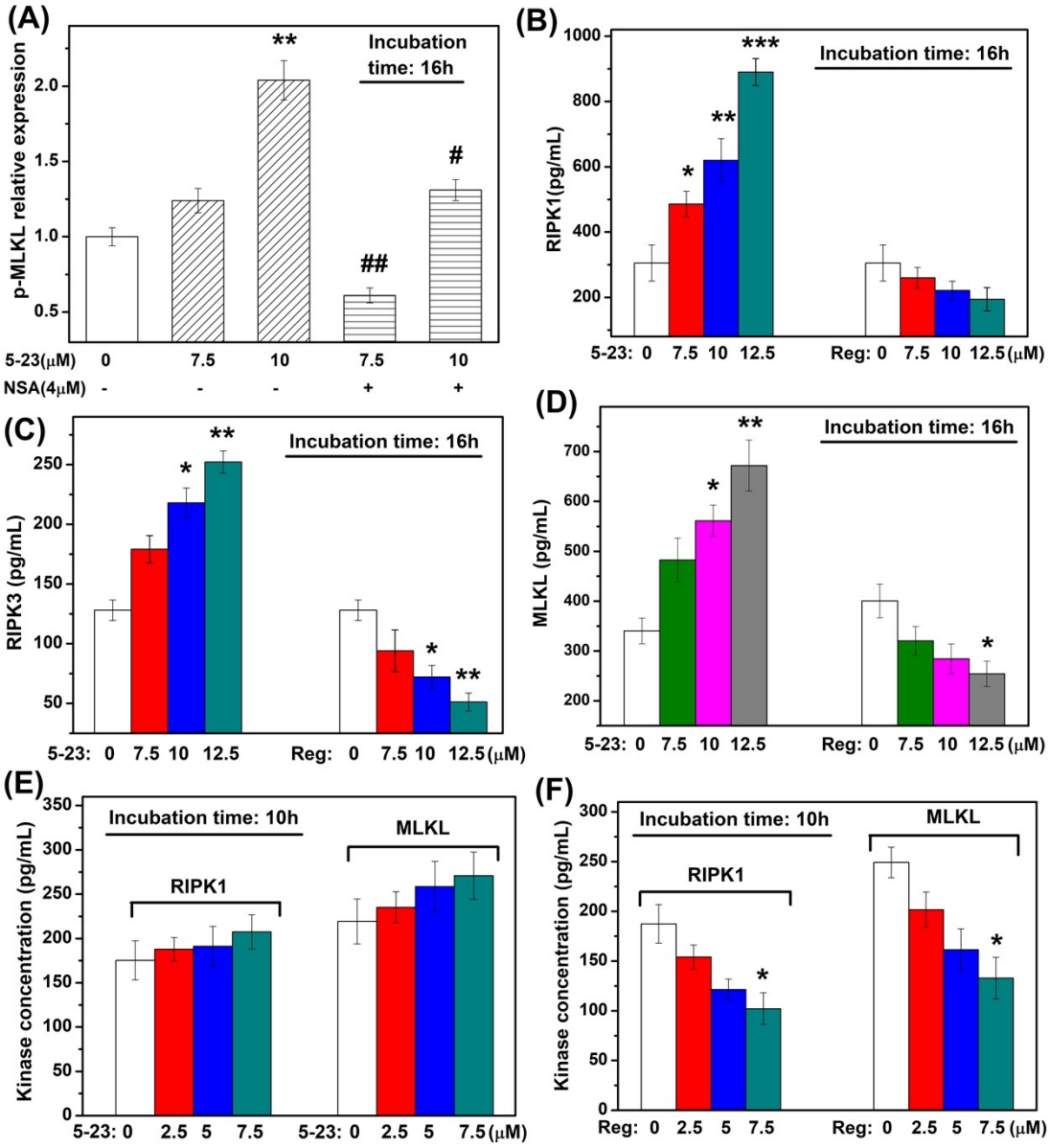


Fig. S4 Intracellular RIPK1, RIPK3, MLKL, and p-MLKL expression and concentration analyses. (A) Expression level changes of p-MLKL after the treatment with **5-23** in the presence (or absence) of necrosulfonamide (NSA). (B–F) Intracellular RIPK1, RIPK3, and MLKL kinase concentration changes before and after the treatment with **5-23** (or Reg) by enzyme-linked immunosorbent assay (ELISA). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus control (concentration: 0 μM). In figure (A), # $p < 0.05$ and ## $p < 0.01$ versus the groups treated only with the same concentration of **5-23** (without the addition of NSA).

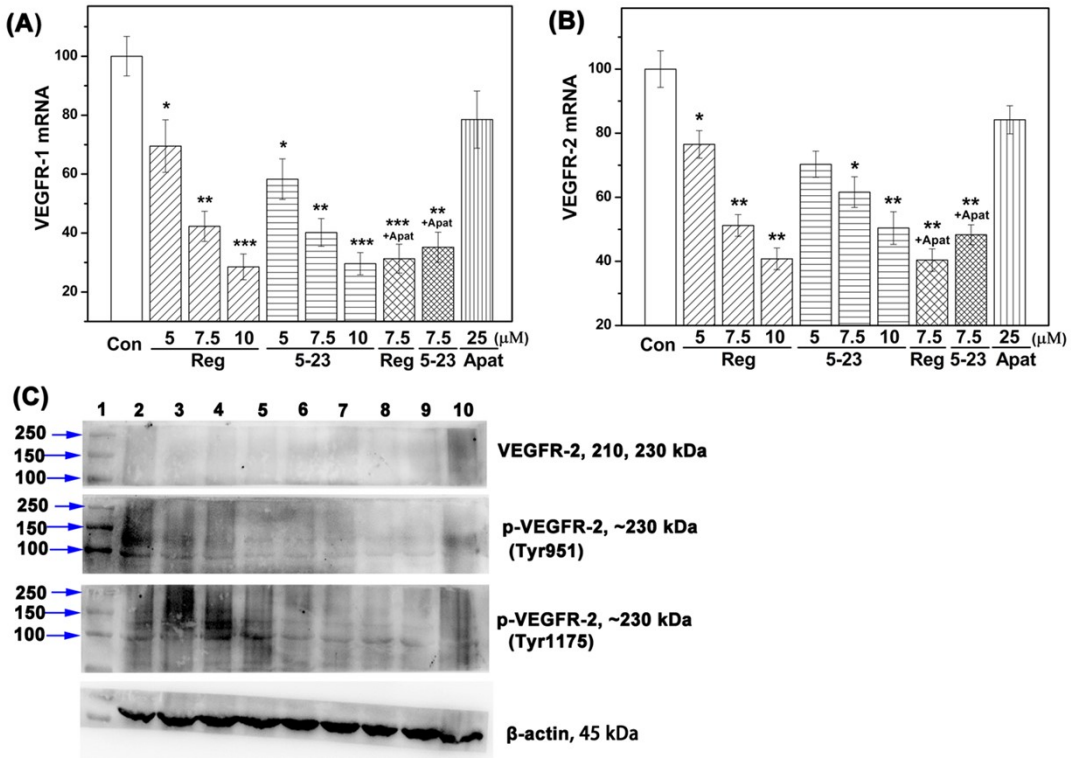


Fig. S5 VEGFR-1 and -2 expressions after T24 cells were treated with **5-23**. Intracellular mRNA expression levels of VEGFR-1 (A) and VEGFR-2 (B) after the treatment with **5-23**. (C) Expressions of VEGFR-2 and p-VEGFR-2 by Western blotting analysis, while their bands were not detected. In Fig. A and B, relative values were normalized to GAPDH for RT-PCR. Each bar represents the mean ± S.D. of three independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus control. Notes of Figure (C), lane 1: Marker; lane 2: Control; lane 3: regorafenib (7.5 μM); lane 4: **5-23** (5 μM); lane 5: **5-23** (7.5 μM); lane 6: **5-23** (10 μM); lane 7: **5-23** (7.5 μM) plus apatinib (12.5 μM); lane 8: **5-23** (7.5 μM) plus apatinib (25 μM); lane 9: **5-23** (10 μM) plus apatinib (25 μM); lane 10: apatinib (25 μM). Incubation time: 16 h.

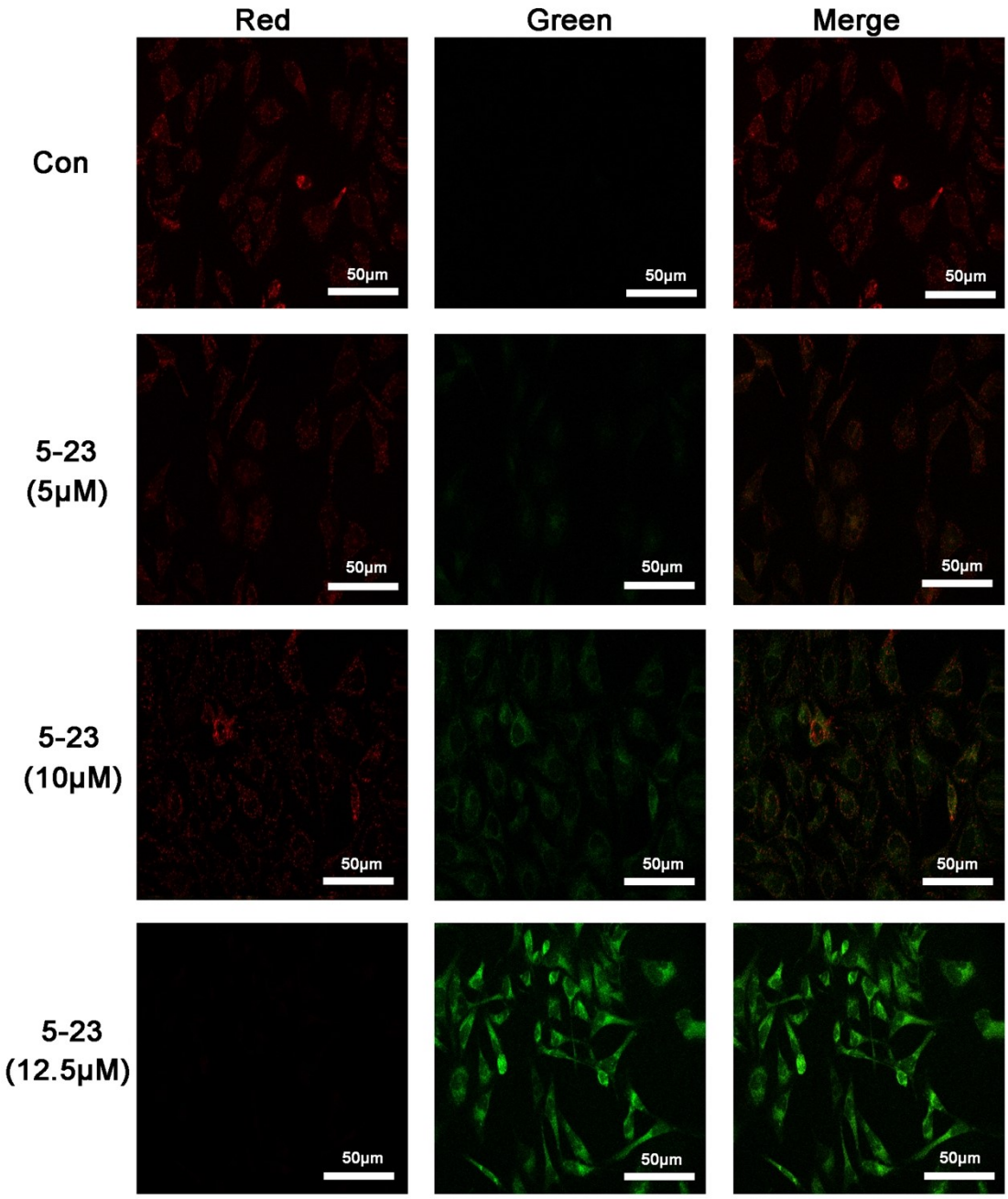


Fig. S6 Compound **5-23** induced loss of mitochondrial membrane potential ($\Delta\Psi_m$). T24 cells were treated with **5-23** for 16 h, harvested, stained with JC-1, and then $\Delta\Psi_m$ was monitored.

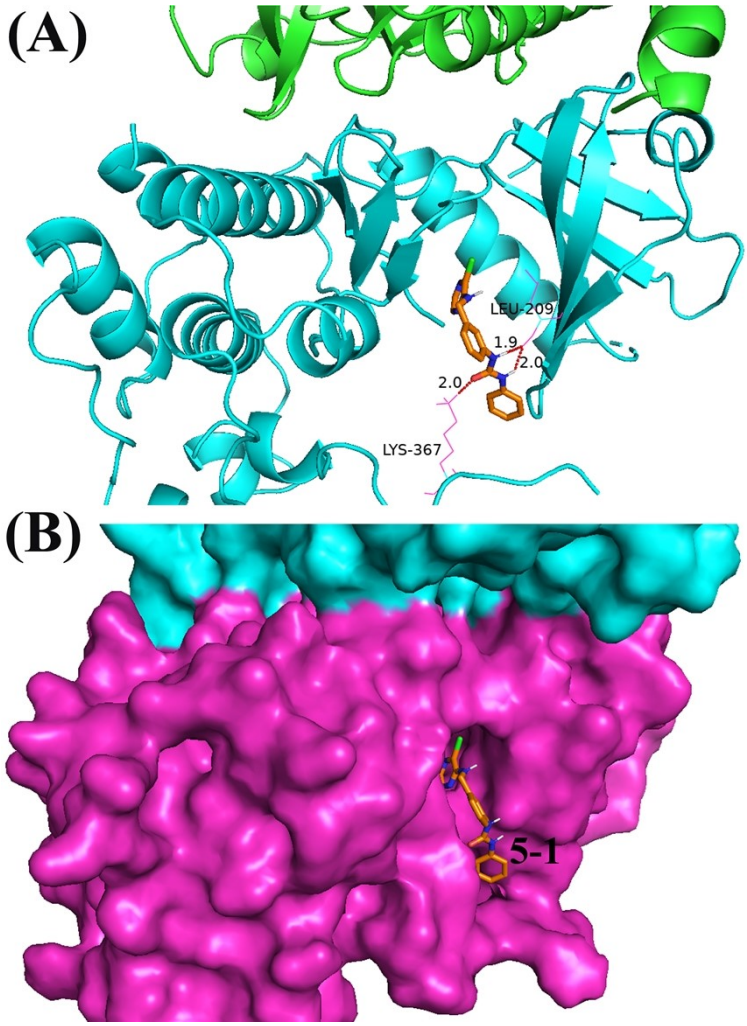


Fig. S7 Binding model of the ligand **5-1** with the receptor MLKL (PDB ID: 6O5Z). (A) Docked conformation of **5-1** when interacted with MLKL. Ligand and key residues are represented as stick models and colored by atom type, whereas the proteins are represented as cartoons. (B) Compound **5-1** embedded in the binding pocket of MLKL. Hydrogen bonds are represented by red dotted lines. For the ligand, white: hydrogen atom; red: oxygen atom; dark blue: nitrogen atom; green: chlorine atom; orange: the backbone and the carbon atoms of **5-1**.

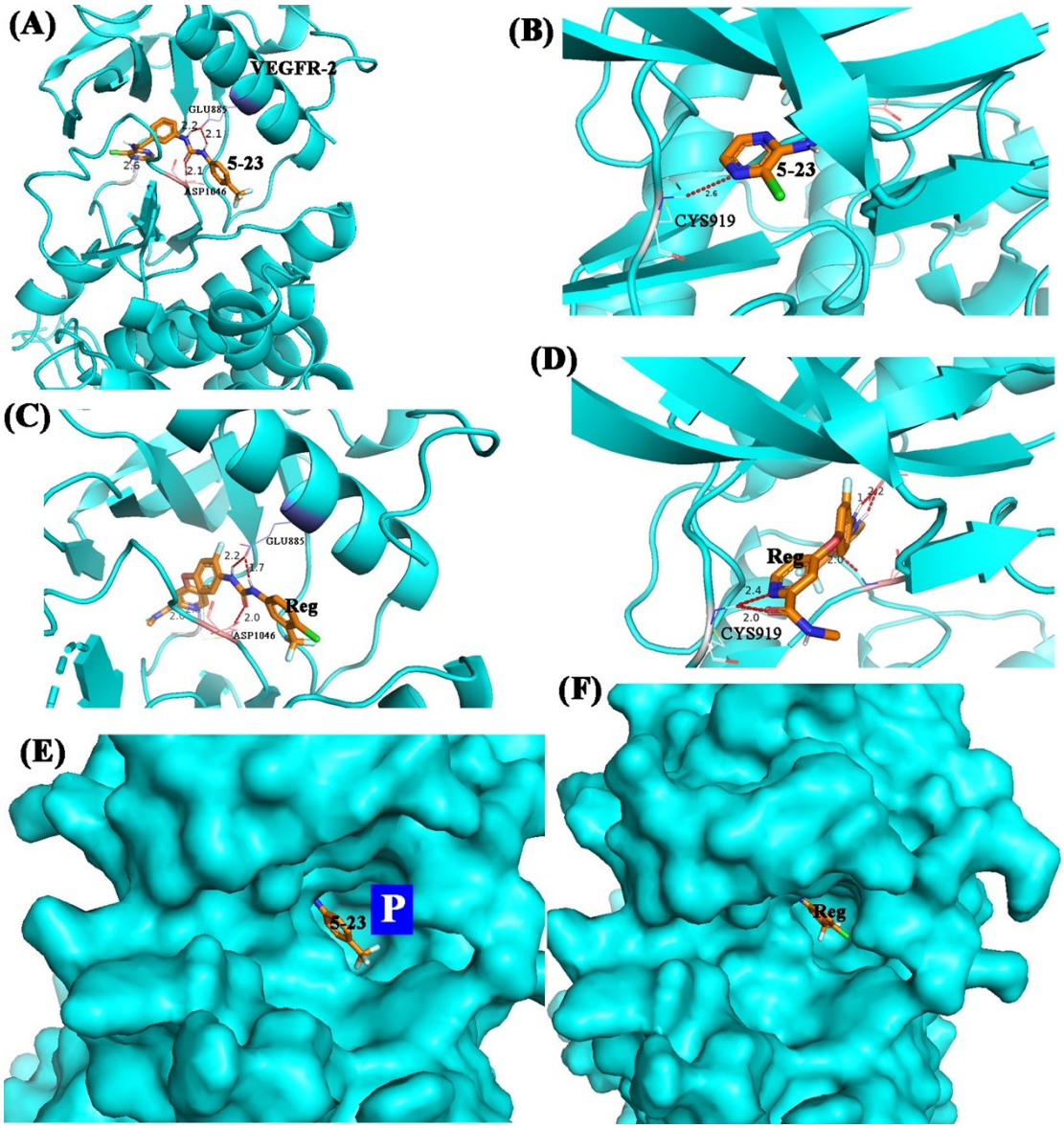
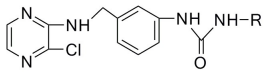


Fig. S8 Binding model of the ligand **5-23** (or Reg) in complex with the receptor VEGFR-2 (PDB ID: 3WZE). Docked conformations of **5-23** (A, B) and Reg (C, D) when interacted with VEGFR-2. Compound **5-23** (E) and Reg (F) embedded in the binding pocket "P" of VEGFR-2.

Table S1–S2

Table S1 Antiproliferative activities of synthesized pyrazinyl-aryl urea derivatives (**5-1**–**5-28**) against NCI-H460 and PC-3 cell lines *in vitro*



Compd.	R	IC_{50} (μM) [a, b]	
		NCI-H460	PC-3
5-1	phenyl	10.62 ± 0.49	38.03 ± 1.71
5-2	2-methylphenyl	6.43 ± 0.39	28.79 ± 1.84
5-3	3-methylphenyl	5.18 ± 0.42	31.29 ± 2.02
5-4	4-methylphenyl	8.19 ± 0.44	29.13 ± 1.77
5-5	2,4-dimethylphenyl	9.82 ± 0.44	26.16 ± 1.39
5-6	3,4-dimethylphenyl	8.79 ± 0.29	21.37 ± 1.05
5-7	2-ethylphenyl	6.59 ± 0.42	17.11 ± 0.62
5-8	2,6-diethylphenyl	11.36 ± 0.74	10.19 ± 0.55
5-9	4-(*tert*-butyl)phenyl	5.73 ± 0.37	18.39 ± 0.97
5-10	2-methoxyphenyl	6.60 ± 0.35	34.13 ± 1.59
5-11	4-methoxyphenyl	8.97 ± 0.92	22.61 ± 1.94
5-12	4-ethoxyphenyl	6.02 ± 0.38	24.03 ± 1.63
5-13	2-fluorophenyl	9.81 ± 0.48	13.17 ± 0.52
5-14	2-chlorophenyl	7.42 ± 0.31	10.13 ± 0.89
5-15	3-chlorophenyl	7.63 ± 0.49	9.41 ± 0.61
5-16	4-chlorophenyl	5.97 ± 0.35	16.12 ± 0.78
5-17	3-chloro-4-fluorophenyl	5.47 ± 0.43	9.03 ± 0.48
5-18	2,4-dichlorophenyl	6.95 ± 0.48	6.92 ± 0.51
5-19	3,4-dichlorophenyl	5.73 ± 0.56	8.23 ± 0.39
5-20	3-chloro-4-methylphenyl	12.77 ± 0.87	12.33 ± 0.61
5-21	2-bromo-4-chlorophenyl	9.34 ± 0.99	11.16 ± 0.77

(Continue)			
5-22	3-trifluoromethylphenyl	5.83 ± 0.41	5.43 ± 0.39
5-23	4-trifluoromethylphenyl	5.71 ± 0.36	4.71 ± 0.29
5-24	4-(*N*,*N*-dimethylamino)phenyl	16.39 ± 0.74	> 40
5-25	4-methylsulfonylphenyl	12.67 ± 0.73	> 40
5-26	α-naphthyl	28.46 ± 1.22	36.19 ± 2.03
5-27	cyclopentyl	> 40	> 40
5-28	cyclohexyl	> 40	> 40
Reg	–	13.75 ± 0.71	15.23 ± 0.86
Gem	–	N.D. [c]	34.13 ± 1.76

[a] Proliferation inhibitory activity was assessed using the MTT assay in cancer cell lines (NCI-H460, PC-3) treated with different concentrations of target compounds and positive reference drugs (regorafenib and gemcitabine) for 48 h, respectively. Data shown are means ± S.D. of four independent experiments.

[b] Compounds with IC_{50} values > 40 μM are considered to be inactive.

[c] N.D. = not determined.

Table S2 Quantitative apoptosis assay of T24 cells treated with compound **5-23** using Annex-V/PI dual staining method [a]

Group	Conc. (μM) [b]	Viable cells (Q1, %)	Early apoptotic cells (Q2, %)	Late apoptotic cells (Q3, %)	Necrotic cells (Q4, %)	Apoptotic cells (Q2 + Q3, %)
Control	0	95.78 ± 2.33	1.66 ± 0.19	1.58 ± 0.38	0.98 ± 0.16	3.24 ± 0.31
Reg	7.5	77.22 ± 3.03	5.84 ± 0.66	12.74 ± 1.36	4.20 ± 0.53	18.58 ± 1.22
Gem	7.5	81.43 ± 2.73	2.14 ± 0.27	11.37 ± 0.91	5.06 ± 0.59	13.51 ± 0.82
5-23	2.5	88.35 ± 2.26	6.02 ± 0.82	2.99 ± 0.39	2.64 ± 0.29	9.01 ± 0.74
5-23	5	82.51 ± 3.47	11.54 ± 1.49	3.29 ± 0.38	2.66 ± 0.47	14.83 ± 1.26
5-23	7.5	72.62 ± 3.25	5.68 ± 0.76	15.73 ± 1.67	5.97 ± 0.65	21.41 ± 1.39
Reg + zVAD	7.5	88.92 ± 1.97	1.92 ± 0.31	5.89 ± 0.74	3.27 ± 0.42	7.81 ± 0.68**
Gem + zVAD	7.5	91.12 ± 2.86	2.57 ± 0.26	3.59 ± 0.43	2.72 ± 0.41	6.16 ± 0.36**
5-23 + zVAD	2.5	92.84 ± 1.71	2.11 ± 0.31	2.74 ± 0.39	2.31 ± 0.25	4.85 ± 0.36*
5-23 + zVAD	5	90.01 ± 2.42	2.07 ± 0.48	5.03 ± 0.79	2.89 ± 0.48	7.10 ± 0.74*
5-23 + zVAD	7.5	87.83 ± 2.89	7.05 ± 0.98	3.46 ± 0.54	1.66 ± 0.23	10.51 ± 0.88**

[a] T24 cells were incubated with compound **5-23** (or positive reference drugs) for 10 h in the presence (or absence) of a pan-caspase inhibitor zVAD (30 μM). The percentage of viable, early apoptotic, late apoptotic, and necrotic cells are presented as mean ± S.D. (n = 3).

[b] Here Conc. refers to the concentration of compound **5-23** or positive reference drugs. * $p < 0.05$ and ** $p < 0.01$ versus the groups in which only the same concentration of compound **5-23** (or positive reference drugs) was added.