Design, Synthesis, And Evaluation Of Cyanopyridines As Anti-Colorectal Cancer Agents Via Inhibiting STAT3 Pathway

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However, to date there are currently no effective STAT3 inhibitors in clinical practice for the treatment of colorectal cancer.

Materials and methods: We screened 27 cyanopyridines for their anticancer activity by cell viability. The HCT-116, RKO, and DLD-1 cell lines were used to evaluate the anti-colorectal cancer effect of 3n. Scratch experiments and colony formation assays were used for the assessment of cell migration and proliferation capacity. Phosphorylated STAT3, STAT3, MCL-1, and Survivin levels were assessed by Western blot analysis.

Results: In this study, we synthesized 27 cyanopyridines and screened their anticancer activities in three human tumor cells, HCT-116, Hela229, and A375. We found that 2-amino-3-cyanopyridine 3n has better anticancer activity with IC50 values in the low micromolar range. Furthermore, 3n significantly inhibited the migration and colony formation of colorectal cancer cells. Mechanistically, 3n inhibited the expression of STAT3 phosphorylation in a dose- and time-dependent manner.

Conclusion: 3n is worth of further investigations toward the discovery of STAT3 inhibitor as a drug candidate for cancer therapy.

Keywords: design, cyanopyridine, colorectal cancer, STAT3, inhibitor

Introduction

Colorectal cancer is one of the common malignant tumors in our digestive system, and the morbidity and mortality rate are increasing year by year. At present, the main treatment for colorectal cancer is surgical treatment, combined with radiotherapy and chemotherapy, but surgery alone may not be cured. Drug treatment of colorectal cancer often has drug resistance, leading to a reduction in colorectal cancer survival rate for cancer treatment. Signal transduction and transcriptional activator (STAT3) is defined as an essential oncogene that regulates cancer cell proliferation, apoptosis, and metastasis. To a large extent, STAT3 is considered to be one of the key oncoproteins and a key therapeutic target, so inhibition of aberrant activation of STAT3 in colorectal cancer may be a promising strategy. However, to date there have been no clinically useful STAT3 inhibitors for the treatment of cancer.

There are many reports on the anti-tumor activity of 2-amino-3-cyanopyridines, but the results are not satisfactory. So far, none of these compounds have entered...
clinical research, and the structure–activity relationship of the system has not been given.13–15 There are relatively few reports on 3-amino-3-cyanopyridine compounds compared to 2-amino-3-cyanopyridine compounds, and there are few studies on the anti-tumor activity of such compounds.16 Our group has previously demonstrated that the aminocyanopyridines exhibit significant antitumor activity against lung cancers in vitro and in vivo.17 So we continued our previous studies to further design, synthesize and evaluate the in vitro antitumor activity of a series of aminocyanopyridines.

Materials And Methods
Antibodies And Reagents
The antibodies against P-STAT3, STAT3, and GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA). The horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Methylthiazolylidiphenyl-tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). The protease phoshatase-inhibitor mixture was obtained from Applygen Technologies (Beijing, People’s Republic of China). Acrylamide (30%), coomassie brilliant blue, tetramethylethylenediamine, tris-glycine, sodium dodecyl sulfate, prestained protein marker, and nonfat dry milk were from Bio-Rad Laboratories.

Chemistry
Reactions were monitored by thin-layer chromatography on silica gel plates (HSGF 254), visualizing with ultraviolet. All the melting points are obtained uncorrected and were taken in a Microscope Melting Point X-6 (Beijing Tech, Beijing, People’s Republic of China). 1H NMR and 13C NMR spectroscopy were determined in Bruker ACANCE III HD-400 and 100 spectrometers (Bruker, Karlsruhe, Germany), respectively. Using DMSO-d6, acetone and CDCl3 solution with TMS as an internal standard. The following abbreviations mean spin multiplicities: singlet (S), doublet (D), triplet (T), and multiplet (M). IR spectra were recorded on a Fourier transform infrared (FTIR) spectrometer MAGNA-IR 550 (Thermo Electron Corporation, MMAS, USA) using KBr (o cm−1). High resolution mass spectrometry (HRMS) were determined on LC-MS QE Focus (Thermo Fisher Scientific, MMAS, USA). Column chromatography was performed on silica gel (200–300 mesh) from Qingdao Ocean Chemicals (Qingdao, Shandong, People’s Republic of China) using distilled petroleum ether and ethyl acetate as mobile phase. Analytical HPLC analyses were performed on Agilent 1260 liquid chromatograph fitted with an Inert-C18 column, with the purity of compounds >95%. All the materials were obtained from commercially available sources and used without further purification, unless otherwise specified. Yields were not optimized.

Cell Culture
Cell culture and human colorectal cell lines HCT-116, RKO, and DLD-1 were purchased from Shanghai cell bank. All of the above cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 media (Thermo Fisher Scientific, Waltham, MA, USA), containing 10% FBS (Gibco, NY, USA) and were maintained at 37°C in a humidified chamber with 5% CO2.

Cell Viability Assay In Vitro
Approximately 1×10⁴ cells, suspended in 1640 medium, were plated onto each well of a 96-well plate and incubated in 5% CO₂ at 37°C for 24 hrs. The test compounds at indicated final concentrations were added to the culture medium and the cell cultures were continued for 72 hrs. Fresh MTT was added to each well at a terminal concentration of 5 mg/mL and incubated with cells at 37°C for 4 hrs. The formazan crystals were dissolved in 150 mL DMSO each well, and the absorbency at 490 nm (for absorbance of MTT formazan) and 630 nm (for the reference wavelength) was measured with the ELISA reader. All the compounds were tested three times in each of the cell lines. The results expressed as IC50 (inhibitory concentration 50%) were the averages of three determinations and calculated by using the GraphPad Prism 6 software.

Colony Formation Assay
The untreated human colorectal cancer cells were plated in 6 well plates at a density of 1000 cells per well. After 7–10 days, the cells were fixed with 4% paraformaldehyde for 10–15 mins, colonies were stained with crystal violet for 15 mins at room temperature.
Cell Migration Assay
RKO and DLD-1 cells were seeded into 6 well plates at a density of 2×10^5 cells per well and cultured to 90% confluence. Then, the culture area was scratched with a sterile crystal pipette tip to make a linear gap in the confluent cell monolayer. The suspended cells were washed gently with PBS. Finally, cells were observed under an inverted microscope, cells were allowed to fill the gap and images of the culture area were captured at 0 hr, 24 hrs and 48 hrs after scratches. Three independent assays were performed.

Western Blot Analysis
The colorectal cells were lysed with RIPA lysis buffer with protease inhibitor (Boster, Wuhan, People’s Republic of China) and the protein concentration was determined after centrifugation at 12,000 rpm at 4°C for 10 mins. Supernatants collected, in order to determine total protein concentration, and protein concentrations assessed using a Bradford protein assay. The PVDF membrane was used to transfer proteins, and the blots were blocked for 90 mins at room temperature with fresh 5% nonfat milk in TBST, then the membrane with the specific primary antibody was incubated at 4°C overnight and at room temperature for 60 mins with the secondary antibody. Finally, the ImageJ software were used to quantify the bands.

Statistical Analysis
Statistics were performed using GraphPad Prism software (GraphPad, San Diego, CA, USA). The differences between sets of data were made using the Student’s t-test. The results are expressed as mean±SE. P<0.05 was statistically significant.

Results
General Procedure For The Compounds
3a-3o (Scheme 1)
A mixture of the substituted acetonaphone (1 mmol), together with the respective benzaldehyde (1 mmol), malononitrile (1 mmol), and ammonium acetate (8 mmol) in toluene (10 mL) was stirred at reflux for 8–12 hrs. After cooling to room temperature, the mixture was diluted ethyl acetate and THF. The organic phase was washed twice with brine, dried over Na2SO4, the filtrate and concentrated under reduced pressure. The residue was suspended in absolute ethanol. The precipitate was collected by filtration and purified by silica gel column chromatography to give the desired product.

2-Amino-6-(4-hydrophenyl)-4-(4-bromophenyl) nicotinonitrile (3a)
White flocule. mp: 304–307°C. 1H NMR (400 MHz, DMSO, δ ppm): 12.91 (s, 1H), 7.71–7.86 (m, 8H), 6.90 (s, 1H). 13C NMR (100MHz, DMSO-d6, δ ppm): 154.03, 149.28, 143.97, 136.83, 133.05, 132.15, 131.24, 123.73, 119.50, 116.15, 115.77, 105.10, 96.30, 95.25. IR (KBr) σ/cm⁻¹: 3456.77, 3349.12, 2205.78, 1620.41, 1594.70, 1574.30, 1545.15, 1519.76, 1492.04, 1456.67, 1363.68, 1231.16, 1172.41, 805.80. HR-ESI-MS (pos. ion mode): m/z=366.0061[M+H]^+ (anal. calcd. for C18H12BrN3O=365.0164).

2-Amino-4, 6-Bis-(4-bromophenyl) nicotinonitrile (3b)
White cottony. mp: 235–237°C. 1H NMR (400 MHz, DMSO-d6, δ ppm): 8.11 (m, 1H), 8.08 (m, 1H), 7.78 (d, J=2.0 Hz, 1H), 7.76 (m, 1H), 7.72 (m, 1H), 7.69 (m, 1H), 7.66 (m, 1H), 7.64 (d, J=2.0 Hz, 1H), 7.32 (s, 1H, cyano-pyridine), 7.11 (s, 2H, amidogen). 13C NMR (101 MHz, DMSO-d6, δ ppm): 161.25, 157.95, 154.37, 137.09, 136.50, 132.17, 132.11, 131.03, 129.77, 124.40, 123.77, 109.47, 87.25. IR (KBr) σ/cm⁻¹: 3494.14, 3389.24, 2205.50, 1607.41, 1580.65, 1543.00, 1489.62, 1454.13, 1392.56, 1341.69, 1364.77, 1073.69, 1009.54, 818.44. HR-ESI-MS (pos. ion mode): m/z=429.9371[M+H]^+ (anal. calcd. for C18H12BrN3O=428.9299).

2-Amino-6-(5-fluro-2-benzyloxy)-4-(4-bromophenyl) nicotinonitrile (3c)
Belge power. mp: 235–237°C. 1H NMR (400MHz, DMSO-d6, δ ppm): 12.97–12.72 (m, 1H), 7.69–7.70 (q, J=2.2 Hz, 1H), 7.64–7.65 (m, 1H), 7.42–7.44 (dd, J=7.7, 1.5 Hz, 2H), 7.26–7.38 (m, 8H), 7.05 (s, 2H), 5.15 (s, 2H, benzlyoxy). 13C NMR (100MHz, DMSO-d6, δ ppm): 161.07, 156.49, 153.52, 153.01, 136.83, 136.49, 132.16, 130.56, 128.94, 128.69, 128.57, 123.58, 117.68, 117.18, 117.13, 115.47, 114.09, 86.74, 71.30. IR (KBr) σ/cm⁻¹: 3470.51, 3329.19, 3199.08, 2919.08, 2206.72, 1648.36, 1572.28, 1544.44, 1501.00, 1452.65, 1426.09, 1253.56, 1231.16, 1172.41, 823.97, 727.52. HR-ESI-MS (pos. ion mode): m/z=473.0539[M+H]^+ (anal. calcd. for C25H13BrFONO=472.0619).
Hz, 2H), 7.49–7.36 (m, 10H), 7.25 (d, J=9.0Hz, 3H), 6.93 (s, 3H), 6.79 (d, J=8.7Hz, 1H, cyanopyridine), 5.18 (d, J=16.3Hz, 4H, benzyloxy).

$^{13}$C NMR (100 MHz, DMSO-d$_6$, $\delta$ ppm): 161.53, 161.05, 158.44, 157.73, 152.50, 137.19, 136.84, 136.77, 132.43, 132.10, 130.52, 128.97, 128.75, 128.58, 128.47, 123.38, 120.00, 113.70, 107.26, 100.96, 85.26, 70.74, 70.00. IR (KBr) $\sigma$/cm$^{-1}$: 3453.09, 3289.25, 3153.39, 2205.43, 1637.18, 1603.08, 1573.97, 1544.55, 1506.81, 1492.87, 1452.29, 1302.11, 1179.88, 1132.98, 1026.70, 822.96, 737.94, 694.70. HR-ESI-MS (pos. ion mode): m/z=564.1107 [M+H]$^+$ (anal. calcd. for C$_{32}$H$_{24}$BrN$_3$O$_2$: 563.1031 (97.3%)).

2-Amino-6-(2, 4-bisfluoro)-4-(4-bromophenyl) nicotinonitrile (3e)
Belge power. mp: 235–238°C. $^1$H NMR (400 MHz, DMSO-d$_6$, $\delta$ ppm): 7.76–7.47 (m, 6H), 7.30–7.26 (m, 1H), 6.99 (d, J=23.6 Hz, 2H), 6.82 (s, 1H, cyanopyridine).$^{13}$C NMR (100 MHz, DMSO-d$_6$, $\delta$ ppm): 154.03, 149.26, 143.96, 136.83, 133.00, 131.22, 123.73, 119.51, 116.14, 115.76, 96.34, 95.28. IR (KBr) $\sigma$/cm$^{-1}$: 3458.51, 3360.33, 3238.35, 2217.85, 1643.64, 1593.06, 1512.73, 1286.61, 1102.15, 813.55. HR-ESI-MS (pos. ion mode): m/z=386.0102[M+H]$^+$ (anal. calcd. for C$_{18}$H$_{10}$BrF$_2$N$_3$: 385.0026).

Scheme 1 The designed route of the 2-amino-cyanopyridines and the chemical structure of the compound 3a-n.

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2-Amino-6-(4-hydroxyphenyl)-4-(3, 4-dimethoxyphenyl) nicotinonitrile (3f)
Yellow power. mp: 155–156°C. $^1$H NMR (400 MHz, DMSO-d$_6$, $\delta$ ppm): 9.91 (s, 1H), 8.01 (d, J=8.5 Hz, 2H), 7.28–7.08 (m, 4H), 3.84 (d, J=5.5 Hz, 6H, methoxy).$^{13}$C NMR (100 MHz, DMSO-d$_6$, $\delta$ ppm): 161.35, 159.96, 158.90, 154.75, 150.39, 149.07, 129.88, 129.42, 128.92, 121.46, 115.83, 112.46, 112.12, 108.51, 85.63, 56.11. IR (KBr) $\sigma$/cm$^{-1}$: 3465.71, 2923.19, 2201.05, 1640.74, 1610.51, 1575.25, 1546.32, 1513.00, 1260.11, 1207.93, 1170.39, 1130.99, 1021.82, 835.97. HR-ESI-MS (pos. ion mode): m/z=346.1202 [M-H]$^-$ (anal. calcd. for C$_{20}$H$_{17}$N$_3$O$_3$: 347.1270).
2-Amino-6-(4-bromophenyl)-4-(3, 4-dimethoxyphenyl) nicotinonitrile (3g)
Brown power. mp: 125–127°C. 1H NMR (400 MHz, DMSO-d6, δ ppm): 8.10 (d, J=6.9 Hz, 2H), 7.71 (d, J=6.9 Hz, 2H), 7.30 (d, J=16.3 Hz, 3H), 7.14 (s, 1H, cyanopyridine), 7.02 (s, 2H), 3.79 (d, J=38.2 Hz, 6H, methoxy). 13C NMR (100 MHz, DMSO-d6, δ ppm): 161.38, 157.59, 155.34, 150.54, 149.08, 137.30, 132.06, 129.75, 129.50, 124.20, 121.61, 117.78, 112.51, 112.11, 109.48, 87.31, 56.13, 56.11. IR (KBr) σ/cm−1: 3470.41, 3321.65, 2214.86, 1632.91, 1400.83, 1303.74, 1103.74, 807.60, 730.25, 688.60. HR-ESI-MS (pos. ion mode): m/z=456.1718[M+H]+ (anal. calcd. for C27H22FN3O2: 455.1645).

2-Amino-6-(2-benzyloxy-5-fluorophenyl)-4-(3, 4-dimethoxyphenyl) nicotinonitrile (3h)
White power. mp: 170–171°C. 1H NMR (400 MHz, DMSO-d6, δ ppm): 7.68 (d, J=9.1 Hz, 1H), 7.41–7.31 (m, 8H), 7.15 (s, 1H, cyanopyridine), 6.97 (dd, J=31.7, 13.3 Hz, 4H), 5.17 (s, 2H, amidogen), 3.84 (s, 3H, methoxy), 3.73 (s, 3H, methoxy). 13C NMR (100 MHz, DMSO-d6, δ ppm): 161.19, 156.27, 154.12, 150.42, 148.98, 136.97, 129.53, 128.88, 128.44, 128.31, 121.27, 117.64, 115.59, 114.19, 112.24, 86.90, 71.20, 59.13, 56.13, 56.03. IR (KBr) σ/cm−1: 3470.41, 3321.65, 3184.79, 2200.00, 1652.56, 1572.13, 1543.83, 1518.63, 1262.81, 1140.83, 1030.74, 807.60, 730.25, 688.60. HR-ESI-MS (pos. ion mode): m/z=456.1718[M+H]+ (anal. calcd. for C27H22FN3O2: 455.1645).

2-Amino-6-(2, 4-bis (benzyloxy)phenyl)-4-(3, 4-dimethoxyphenyl) nicotinonitrile (3i)
Faint yellow power. mp: 146–148°C. 1H NMR (400 MHz, DMSO-d6, δ ppm): 8.70 (d, J=8.7 Hz, 1H), 7.51–7.47 (m, 2H), 7.42 (t, J=7.4 Hz, 4H), 7.38–7.28 (m, 5H), 7.13 (d, J=2.1 Hz, 1H), 6.98 (d, J=8.4 Hz, 1H), 6.92 (d, J=2.3 Hz, 1H), 6.88–6.81 (m, 1H), 6.81 (m, 3H), 5.19 (d, J=2.9 Hz, 4H, benzyloxy), 3.84 (s, 3H, methoxy), 3.72 (s, 3H, methoxy). 13C NMR (100 MHz, DMSO-d6, δ ppm): 161.32, 161.12, 158.30, 157.49, 153.62, 150.26, 148.91, 137.23, 137.00, 132.40, 129.84, 128.96, 128.91, 128.33, 121.16, 120.49, 118.05, 113.84, 112.80, 107.24, 101.13, 85.56, 70.61, 69.99, 56.10, 56.00. IR (KBr) σ/cm−1: 3454.83, 3361.04, 2920.39, 2206.18, 1634.03, 1603.86, 1560.54, 1514.22, 1454.06, 1364.47, 1257.66, 1177.78, 1132.43, 1022.66, 822.00, 729.31, 693.45. HR-ESI-MS (pos. ion mode): m/z=476.0593[M+H]+ (anal. calcd. for C25H17BrF3N3O: 475.0519 [97.3%]).
2-Amino-6-(2, 4-bis (benzyloxy) Phenyl)-4-(2-bromophenyl) nicotinonitrile (3n)
White powder. mp: 171–173°C. \(^1\)H NMR (400 MHz, DMSO-d6, δ ppm): 7.93(d, J=8.7 Hz, 1H), 7.77 (s, 1H), 7.41 (m, 9H), 7.23 (d, J=17.5 Hz, 5H), 6.93 (s, 3H), 6.80 (d, J=7.3 Hz, 1H), 5.18 (d, J=11.1 Hz, 4H, benzyloxy), 4.35 (d, J=3.2 Hz, 2H). \(^{13}\)C NMR (100 MHz, DMSO-d6, δ ppm): 160.35, 158.05, 156.39, 155.70, 154.30, 153.40, 152.87, 151.34, 130.69, 128.76, 128.42, 128.23, 127.82, 121.63, 117.94, 117.71, 117.28, 117.04, 116.41, 115.75, 115.67, 114.71, 88.91,71.09. IR (KBr) σ/cm\(^{-1}\): 3490.49, 3363.26, 2218.47, 1623.93, 1569.13, 1599.12, 1420.92, 1261.12, 1205.33, 1025.16, 780.48, 763.31, 753.71. HR-ESI-MS (pos. ion mode): m/z=564.1107[M+H]+ (calcd. for C\(_{32}\)H\(_{24}\)BrN\(_3\)O\(_2\): 563.1031 (97.3%)).

General Procedure For The Compounds 4a-4o (Scheme 2)
A mixture of the substituted acetophenone (1 mmol), together with the respective benzaldehyde (1mmol), ethylcyanoacetate (1mmol) and ammonium acetate (8mmol) in toluene (10mL) was stirred at reflux for 8–12 hrs. After cooling to room temperature, the mixture was diluted ethyl acetate and THF. The organic phase was washed twice with brine, dried over Na\(_2\)SO\(_4\), the filtrate and concentrated under reduced pressure. The residue was suspended in absolute ethanol. The precipitate was collected by filtration and purified by silica gel column chromatography to give the desired product.

4, 6-bis(4-bromophenyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (4a)
Yellow power. mp: 168–170°C. \(^1\)H NMR (400 MHz, DMSO-d6, δ ppm): 10.44 (s, 1H), 8.08 (d, J=8.0 Hz, 2H), 7.84 (d, J=7.4 Hz, 2H), 7.66 (s, 3H), 6.90 (d, J=8.0 Hz, 2H). \(^{13}\)C NMR (400 MHz, DMSO-d6, δ ppm): 187.47, 162.77, 141.80, 134.69, 132.30, 131.73, 131.10, 129.48, 124.09, 123.41, 115.87. IR (KBr) σ/cm\(^{-1}\): 3215.87, 2368.47, 1654.30, 1598.86, 1573.37, 1513.99, 1486.06, 1334.97, 1279.95, 1225.22, 1167.92, 812.66. HR-ESI-MS (pos. ion mode): m/z=367.0077[M+H]+ (anal. calcd. for C\(_{18}\)H\(_{11}\)BrN\(_2\)O\(_2\): 366.0004).
6-(2-(benzoxyl)-5-fluorophenyl)-4-(4-bromophenyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (4c)

Yellow needle power. mp: 118–120°C. 1H NMR (400 MHz, DMSO-d6, δ ppm): 7.71 (m, 1H), 7.54 (d, J = 8.5 Hz, 3H), 7.33 (m, 1H), 7.21 (s, 1H), 6.99 (d, J = 8.4 Hz, 2H), 6.73 (s, 2H), 5.14 (s, 2H). 13C NMR (400 MHz, DMSO-d6, δ ppm): 167.81, 166.22, 158.44, 154.72, 153.41, 150.78, 140.69, 137.27, 136.03, 131.51, 129.89, 128.90, 128.76, 121.60, 117.34, 116.91, 115.59, 106.06, 71.22. IR (KBr) σ/cm⁻¹: 3415.91, 3272.92, 3173.49, 2985.27, 2247.60, 1615.98, 1572.50, 1491.94, 1282.43, 1250.94, 1192.61, 1108.16, 1014.47, 810.47, 735.28. HR-ESI-MS (pos. ion mode): m/z=475.0945[M + H]⁺ (calcd. for C₂₅H₁₈BrF₃N₂O₂: 474.0379).

6-(2-benzyloxy)5-fluorophenyl)-4-(4-bromophenyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (4d)

Faint yellow power. mp: 165–168°C. 1H NMR (400 MHz, DMSO-d6, δ ppm): 7.91 (d, J = 8.7 Hz, 1H), 7.41 (m, 1H), 7.13 (s, 1H), 6.97 (d, J = 8.3 Hz, 2H), 6.70 (s, 2H), 5.17 (d, J = 20.0 Hz, 4H, benzoxyl). 13C NMR (100 MHz, DMSO-d6, δ ppm): 167.98, 161.14, 158.59, 158.25, 150.78, 140.69, 137.27, 136.94, 132.24, 131.40, 129.87, 128.95, 128.92, 128.58, 128.44, 128.34, 121.35, 115.38, 107.15, 100.98, 70.64, 69.98. IR (KBr) σ/cm⁻¹: 3422.83, 3266.96, 3168.02, 2918.61, 2360.65, 1680.43, 1603.46, 1571.60, 1488.09, 1258.68, 1186.16, 1100.30, 1021.82, 1011.92, 818.95, 735.55, 693.30. HR-ESI-MS (pos. ion mode): m/z=563.0972[M + H]⁺ (calcd. for C₃₃H₂₃BrN₂O₃: 562.0892).
6-(2,4-bis(benzyloxy)phenyl)-4-(3,4-dimethoxyphenyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (4i)
Faint yellow powder. mp: 178–180°C. 1H NMR (400 MHz, DMSO-d6, δ ppm): 7.87 (d, J=7.8 Hz, 1H), 7.48–7.33 (m, 10H), 7.20 (s, 1H), 6.90 (s, 2H), 6.78–6.73 (m, 2H), 6.57 (d, J=6.0 Hz, 1H), 6.49 (s, 1H), 5.18 (s, 4H), 3.80 (s, 3H), 3.66 (s, 3H). 13C NMR (400 MHz, DMSO-d6, δ ppm): 168.65, 160.85, 158.13, 158.03, 155.71, 148.77, 137.11, 133.64, 128.95, 128.87, 128.41, 128.32, 128.24, 124.80, 124.74, 120.18, 112.11, 101.11, 70.51, 69.95. IR (KBr) σ/ cm⁻¹: 3427.79, 2961.41, 2922.03, 2360.35, 1679.82, 1603.49, 1562.38, 1508.49, 1457.52, 1260.44, 1104.97, 1025.11, 815.30. HR-ESI-MS (pos. ion mode): m/z=545.2073[M+H]⁺ (calcd. for C34H28N2O5: 544.998).

6-(2, 4-difluorophenyl)-4-(3, 4-dimethoxyphenyl)-2-oxo-1, 2-dihydropyridine-3-carbonitrile (4j)
Yellow power. mp: 253–255°C. 1H NMR (400 MHz, DMSO-d6, δ ppm): 7.85 (s, 1H), 7.42 (t, J=12.5 Hz, 1H), 7.25 (s, 3H), 7.11 (s, 1H), 6.65 (d, J=29.9 Hz, 1H), 5.31 (s, 1H), 3.83 (s, 6H), 3.82 (s, 6H). 13C NMR (100MHz, DMSO-d6, δ ppm): 150.73, 148.98, 133.00, 132.99, 132.93, 132.89, 121.58, 112.52, 112.30, 112.25, 112.09, 105.11. IR (KBr) σ/ cm⁻¹: 3440.10, 3055.13, 2919.16, 2847.54, 2430.58, 2215.93, 1656.98, 1617.30, 1520.05, 1471.72, 1023.85, 801.99. HR-ESI-MS (pos. ion mode): m/z=369.1045 [M–2H]⁺ (calcd. for C20H12F2N2O2: 367.1132).

4-(2-bromophenyl)-6-(4-hydroxyphenyl)-2-oxo-1, 2-dihydropyridine-3-carbonitrile (4k)
Brown power. mp: 132–135°C. 1H NMR (400 MHz, DMSO-d6, δ ppm): 9.88 (s, 1H), 7.98 (t, 1H), 7.95 (t, 1H), 7.67–7.55 (m, 1H), 7.46–7.41 (td, J=7.5, 1.2 Hz, 1H), 7.34–7.29 (m, 2H), 7.13 (s, 2H), 6.84 (s, 2H), 6.82 (d, J=1.9 Hz, 1H). 13C NMR (100 MHz, DMSO-d6, δ ppm): 159.83, 158.23, 152.96, 143.30, 132.26, 131.17, 129.83, 129.45, 129.15, 128.79, 127.80, 121.70, 115.86, 110.41, 103.37. IR (KBr) σ/ cm⁻¹: 3470.22, 3413.54, 3369.47, 1686.21, 1599.93, 1570.73, 1516.70, 1360.58, 1282.87, 1230.64, 1169.10, 1102.44, 823.99, 755.09. HR-ESI-MS (pos. ion mode): m/z =367.0089[M+H]⁺ (calcd. for C16H11BrN2O2: 366.0004).

6-(2, 4-bis (benzyloxy) Phenyl)-4-(2-bromophenyl)-2-oxo-1, 2-dihydropyridine-3-carbonitrile (4n)
White powder. mp: 170–172°C. 1H NMR (400 MHz, DMSO-d6, δ ppm): 7.95 (d, J=8.3 Hz, 1H), 7.64 (d, J=6.3 Hz, 1H), 7.38 (dd, J=61.3, 24.8 Hz, 12H), 7.07 (s, 4H), 6.91 (s, 1H), 6.80 (d, J=7.9 Hz, 1H), 5.17 (d, J=19.3 Hz, 4H). 13C NMR (100 MHz, DMSO-d6, δ ppm): 159.39, 152.13, 142.80, 136.91, 132.26, 129.58, 129.48, 128.90, 128.77, 128.20, 127.81, 121.56, 117.56, 117.34, 117.23, 116.98, 116.20, 115.65, 115.57, 104.85. IR (KBr) σ/ cm⁻¹: 3422.71, 3263.89, 3165.39, 2922.32, 2337.22, 1617.43, 1602.93, 1568.64, 1259.88, 1185.94, 1107.73, 1024.55, 817.10, 732.12, 693.23. HR-ESI-MS (pos. ion mode): m/z=563.0972[M+H]⁺ (calcd. for C25H16BrN2O2: 562.0892).
4-(2-bromophenyl)-6-(2,4-difluorophenyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (4o)

White power. mp: 300–302°C. 1H NMR (400 MHz, DMSO-d6, δ ppm): 13.09 (s, 1H), 7.80 (d, J=15.4 Hz, 2H), 7.55–7.47 (m, 4H), 7.28 (s, 1H), 6.61 (s, 1H). 13C NMR (100 MHz, DMSO-d6, δ ppm): 137.52, 133.47, 133.06, 132.96, 132.01, 130.47, 128.62, 120.87, 115.87, 112.95, 105.77, 105.50, 105.24. IR (KBr) σ/cm−1: 3443.52, 2778.95, 2221.46, 1650.66, 1614.21, 1506.77, 1269.52, 1226.79, 1110.34, 949.56, 853.61, 756.74. HR-ESI-MS (pos. ion mode): m/z=386.9782[M+H]+ (calcd. for C18H9BrF2N2O: 385.9866).

Screening Of Cyanopyridines Against Human Cancer Cells In Vitro

First, all the synthesized cyanopyridines were evaluated for their antiproliferative activity against a variety of cancer cells by the MTT assay in vitro (Tables 1–2). Among the cyanopyridines 3n exhibited potent inhibitory activity on all HCT-116, Hela, and A375 cells with the IC50 values of 10.50, 14.27 and 4.61 μM, respectively, which was the more potent compound for further study.

From the preliminary structure–activity relationships, we may conclude that introduction of benzyloxy group to the 1-position and 2-position of phenyl ring slightly enhance the cytotoxic activity, meanwhile bromine substituent to the 6-position on phenyl ring even had decreased activity compared to 3n. Furthermore, the compound 3n with 2-amino substituent was more active than 2-oxo congen 4n.

Active Compounds 3n Inhibited Migration And Colony Formation Of Colorectal Cancer Cells

Moreover, DLD-1 and RKO cells were treated with different concentrations of 3n to evaluate the effect of 3n on colorectal cancer cells migration (Figure 1A). The migration ability of cells treated with 3n was significantly reduced, which highlighted the critical role of 3n in inhibition of colorectal cancer cell migration. In addition, to observe the effect of 3n on the proliferation of colorectal cancer cells, we performed colony formation assays. Similar effects were observed in colony formation assays, 3n remarkably reduced the colony formation capacity in HCT116, RKO, and DLD-1 cancer cell lines (Figure 1B).

The Compounds 3n Inhibited The Phosphorylation Of STAT3

Aminocyanopyridines can inhibit cell proliferation and induce cell death in human cancer cells by inhibiting the STAT3 pathway.17 We next validated the effects of 3n on STAT3 signaling in three colorectal cancer cells. As shown in Figure 2A, STAT3 phosphorylation was inhibited in a time-dependent manner with a 40 μM concentration of 3n, which significantly inhibited STAT3 phosphorylation at 24
hrs, but had no effect on total STAT3 levels in the three cell lines. Moreover, incubation of HCT116 with a concentration gradient of 3n (10, 20, and 40 μM) of 3n showed that the cell migration was significantly slowed down with increasing concentration. Wound healing assay results from RKO and DLD-1 were calculated ($^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$). (B) For the colony formation assay, the number of colonies was counted 6–8 days after colorectal cancer cells were incubated and treated with various concentrations for 3n, and DMSO (1 μL) was added as a negative control and then stained with crystal violet.

Figure 2B shows that the downstream protein levels of STAT3 and found that 3n also inhibits the expression of downstream target proteins, such as Survivin and MCL-1 (Figure 2D).
Figure 2. The compound 3n inhibited STAT3 phosphorylation in colorectal cancer cells. (A) The three colorectal cells were exposed to 40 μM 3n for different time periods (0, 2, 4, 8, 12, and 24 hrs). Total protein was extracted, and the expression levels of P-STAT3, STAT3, and GAPDH proteins were detected by Western blot analysis. (B) Western blot analysis of STAT3 pathway associated proteins in colorectal cancer cells exposed to various concentrations (10, 20, and 40 μM) of 3n for 24 hrs. Representative pictures from three independent experiments are shown. (C) Human colon cancer cells were pretreated with 3n (10, 20, and 40 μM) and napabucasin (napa 1 μM) for 24 hrs and then stimulated with IL6 (25 ng/mL) for 30 mins. (D) The effect of 3n on Survivin and MCL-1 was analyzed by Western blot in RKO cells. (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Abbreviations: DMSO, dimethyl sulfoxide; STAT3, signal transducer and activator of transcription 3; P-STAT3, phosphorylated STAT3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MCL-1, myeloid cell leukemia-1.
Discussion
Colorectal cancer remains the leading cause of cancer deaths worldwide, despite extensive research and progress in screening and treatment.1,18,19 In addition, to date, there are no convincing agents that can significantly improve the survival rate of human colorectal cancer.5 STAT3 is considered to be an essential oncogene in the development of colorectal cancer and is thought to be carcinogenic in the development of colorectal cancer.20 In future studies, finding a suitable STAT3 inhibitor to inhibit abnormal activation of STAT3 in colorectal cancer cells remains a promising strategy to address this problem.21,22

In this study, we designed two new series of compounds with 2-amino-3-cyanopyridine chalcone and 3-cyano-2-pyridinone skeleton chalcone. Three kinds of human tumor cells HCT-116, Hela229, and A375 were screened for biological activity against proliferation. The results showed that 3n can effectively inhibit the proliferation and migration ability of colorectal cancer cells. Further studies confirmed that STAT3 phosphorylation was inhibited in a time- and dose-dependent manner after exposure to 3n. These results indicate that aminohydroxypyridine compound 3n can act as an inhibitor of STAT3, thereby inhibiting tumor growth and inducing apoptosis in colorectal cancer. 3n is a potent STAT3 inhibitor and may be a potential drug candidate for colorectal cancer therapy. Also, of course, 3n can also serve as lead compounds for optimization to speed the development of drugs selectively targeting the IL-6/STAT3 cancer signaling pathway. Additionally, the pharmacokinetics, pharmacodynamics, and toxicity of 3n will be further comprehensively evaluated. Combination therapy may enhance the curative effects and reduce the therapeutic concentration of chemotherapeutic drugs.23,24 We also plan to investigate the combination of 3n and other clinical cancer drugs to improve the antitumor potency of 3n and verify its effect on colorectal cancer.

Conclusion
In summary, a novel series of cyanopyridines and aminocyanopyridines were designed, synthesized, and biologically evaluated. The most potent compound 3n exhibited remarkable inhibitory activity on HCT116, Hela, and A375 cells. Furthermore, 3n significantly inhibits cell colony formation and migration of colorectal cancer cells. Mechanistically, 3n decreased protein expression level of P-STAT3Y. Together, 3n is worth of further investigations toward the discovery of STAT3 inhibitor as a drug candidate for cancer therapy.

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Disclosure
The authors report no conflicts of interest in this work.

References


