Bioorganic & Medicinal Chemistry Letters 22 (2012) 6224–6228

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Design and synthesis of diarylamines and diarylethers as cytotoxic antitumor agents

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ARTICLE INFO

Article history:
Received 4 June 2012
Revised 26 July 2012
Accepted 1 August 2012
Available online 9 August 2012

Keywords:
Diarylamines
Diarylethers
Cytotoxicity
Mer tyrosine kinase

ABSTRACT

Based on a shared structural core of diarylamine in several known anticancer drugs as well as a new cytotoxic hit 6-chloro-2-(4-cyanophenyl)amino-3-nitropyridine (**7**), 30 diarylamines and diarylethers were designed, synthesized, and evaluated for cytotoxic activity against A549, KB, KB-vin, and DU145 human tumor cell lines (HTCL). Four new leads **11e**, **12**, **13a**, and **13b** were discovered with GI_{50} values ranging from 0.33 to 3.45 μM. Preliminary SAR results revealed that a diarylamine or diarylether could serve as an active structural core, *meta*-chloro and *ortho*-nitro groups on the A-ring (either pyridine or phenyl ring) were necessary and crucial for cytotoxic activity, and the *para*-substituents on the other phenyl ring (B-ring) were related to inhibitory selectivity for different tumor cells. In an investigation of potential biological targets of the new leads, high thoughput kinase screening discovered that new leads **11e**, **12** and **13b** especially inhibit Mer tyrosine kinase, a proto-oncogene associated with munerous tumor types, with IC_{50} values of 2.2–3.0 μM. Therefore, these findings provide a good starting point to optimize a new class of compounds as potential anticancer agents, particularly targeting Mer tyrosine kinase.

Cancers are complex diseases that involve uncontrolled multiplication and spread (metastasis) of abnormal cells. Over the past decade, advances in our understanding of the molecular biology of tumor cells have greatly promoted the discovery of small-molecule antitumor drugs targeting distinct proteins in signal transduction pathways. Imatinib (**1**, Gleevec), the first drug that specifically inhibited Abl kinase,[1] is used in treating chronic myelogenous leukemia (CML), gastrointestinal stromal tumors (GISTs) and other cancers. Gefitinib (**2**, Iressa)[2] and Erlotinib (**3**, Tarceva),[3] both epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, were approved for treatment of metastatic non-small cell lung cancer, pancreatic cancer, and several other types of cancer. Sorafenib (**4**, Nexavar)[4] was used against advanced renal cancer. Recently, two kinase inhibitor drugs nilotinib (**5**) and dasatinb (**6**) were approved to treat chronic myeloid leukemia (CML). Even though these drugs and some clinical candidates have provided better treatments for cancer patients, the chemotherapeutic therapy is still largely unsatisfactory and there remains an urgent need to develop innovative agents and/or novel combinations to improve current therapeutic effectiveness.

In an analysis of the structures of the described tyrosine kinase inhibitors **1**–**6**, as well as other clinical candidates, we discovered that most of them share a basic diarylamine or diarylether functionality as shown in red in Figure 1. Therefore, we initially synthesized the hit compound **7** [6-chloro-2-(4-cyanophenyl) amino-3-nitropyridine] with a similar basic structure core to serve as the design template. Compound **7** showed significant cytotoxic activity against a panel of human tumor cell lines (HTCL), including A549 (human lung cancer), KB (nasopharyngeal carcinoma), KB-vin (vincristine-resistant KB subline), and DU145 (prostate cancer) cell lines, with low micromolar GI_{50} values of 2.11 to 6.12 μM. Accordingly, **7** was modified by the strategy shown in Figure 2. We first changed substituents R^1 on the B-ring and R^2 on the A-ring, and subsequently conducted isosteric replacements for the A-ring and the linker (Y). Totally, 30 diarylamines (**9a**–**k**, **10a**–**c**, **11a**–**e**) and diarylethers (**12**, **13a**–**e**, **14a**–**f**) were synthesized and evaluated against a human tumor cell line (HTCL) panel, including A549,

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http://dx.doi.org/10.1016/j.bmcl.2012.08.014

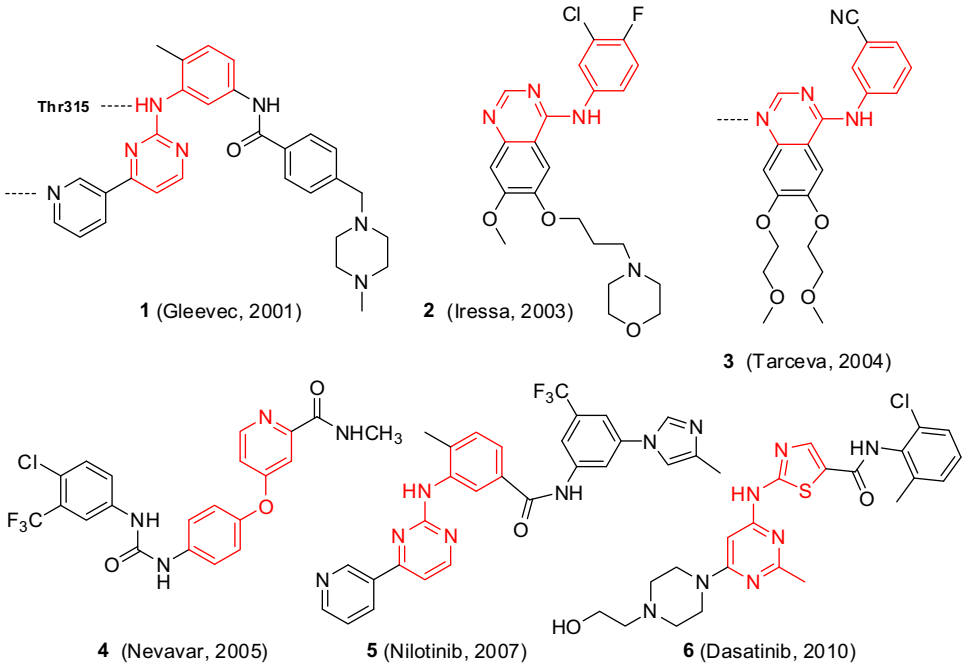


Figure 1. Anticancer drugs **1**–**6** targeting different kinases.

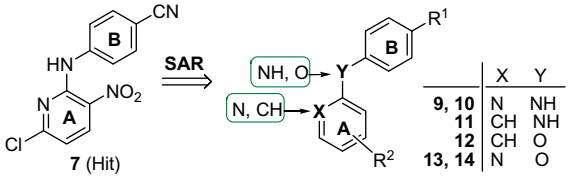


Figure 2. Structural modification and target compounds **9**–**14**.

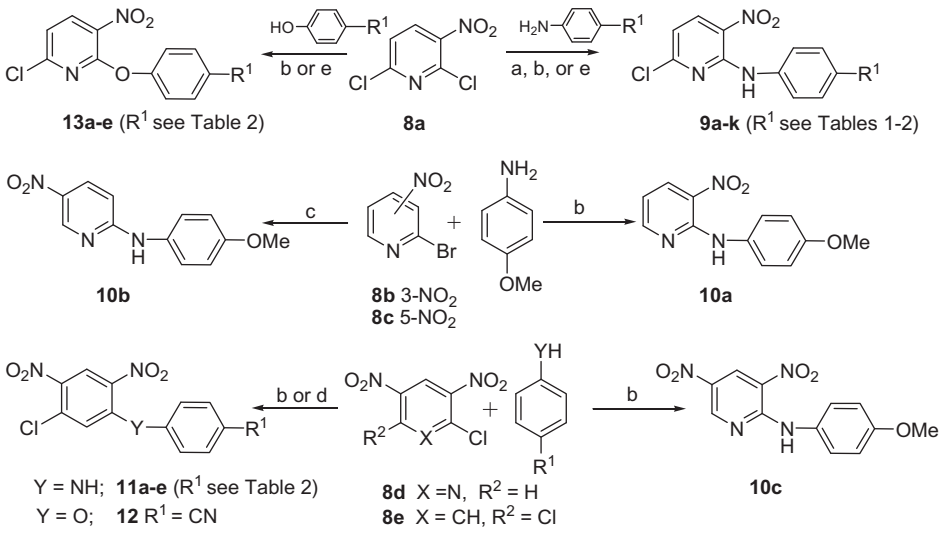
KB, KBvin, DU145, and K652. Furthermore, active new leads were screened against 72 kinases in a high throughput screening platform to explore potential biologic targets. Herein, we present chemical synthesis of the new diarylamines and diarylethers, their cytotoxic activities, SAR results, and potential biologic target of new leads.

Chemistry

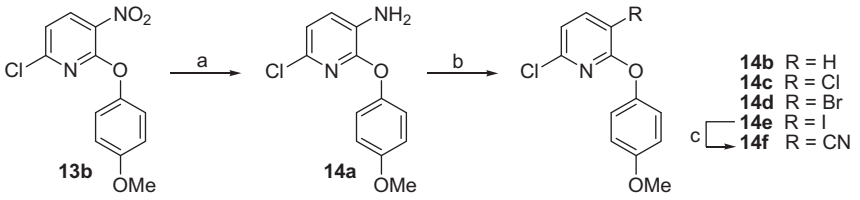
Target compounds **9**–**13** were synthesized as shown in Scheme 1. Halogenated mono- or di-nitropyridines (**8a**–**8d**) and dinitrobenzene (**8e**), and various 4-substituted anilines or phenols were commercially available. Because the nucleophilicity of a substituted aniline or phenol affects the coupling reaction, target compounds were synthesized under different reaction conditions (see Scheme 1). The substitution reactions of 2,5-dichloro-3-nitro-pyridine **8a** with a nucleophilic aniline or phenol took place preferentially at the chloride on the *ortho*-position to the nitro group[5] to give **9a**–**k** and **13a**–**e**. The coupling between **8a** and anilines substituted with an electron-donating group(s) proceeded easily in the presence of $NaHCO_3$ in EtOH at room temperature (Method A, see **9a**–**e**).[6] A microwave-assisted method (Method B)[7] was also applied to the coupling reaction for synthesizing compounds **9f**–**g**, **9i**–**k**, **10a**, **10c**, **12**, and **13e** in the presence of anhydrous potassium carbonate in *t*-BuOH at 120–190 °C for 10–40 min, achieving moderate to high yields. However, the coupling reaction of an aniline with a strong electron-withdrawing carboxylic group (CO_2H) was more difficult, even at higher temperature over longer times, and **9j** was obtained in a lower 21% yield. The synthesis of 5-nitropyridine analog **10b** was accomplished by using the polar aprotic solvent DMSO and K_2CO_3 at 110 °C for 4 h (Method C).[8] With DMF as the solvent, diphenylamines **11a**–**11e** were produced easily in high yields of 80–94% in the presence of Et_3N or *t*-BuOK at room temperature for 30 min (Method D).[9,10] Moreover, mild conditions were also used to prepare **9h** and **13a**–**d** in moderate to high yields (65–91%) in the presence of K_2CO_3 in *t*-BuOH at room temperature for 12–24 h (Method E).[11] Scheme 2 illustrates the syntheses of compounds **14a**–**f** with different substituents at the 3-position on the pyridine ring. The 3-nitro group in **13b** was first reduced to an amino group in **14a** by using literature methods.[12] Subsequently, diazotization by $NaNO_2$ in the presence of H_2SO_4, HCl, or HBr and treatment of the resulting diazonium salt with Cu_2O/EtOH, CuCl, CuBr, or KI, respectively, afforded the corresponding **14b** and halogenated compounds **14c**–**e**, that is, the diazo group was replaced by H, Cl, Br, or I, respectively. Subsequently, the iodo compound **14e** was treated with cuprous cyanide to produce the cyano compound **14f** in a 96% yield. All target compounds were identified by NMR and MS spectroscopic data.[13]

The 30 newly synthesized diarylamines (**9**–**11** series) and diarylethers (**12**–**14** series) were initially screened for cytotoxic activity against a HTCL panel (A549, KB, KB-vin, and DU145 cancer cell lines)[14] with paclitaxel and **7** serving as reference compounds. The in vitro anticancer activity (GI_{50}) was determined using the established sulforhodamine B (SRB) method.[15] Target compounds and their biological data are summarized in Tables 1 and 2. Among them, the four most potent new compounds, **11e**, **12**, **13a**, and **13b**, showed lower micromolar GI_{50} values of 0.33–3.45 μM against the HTCL panel and were more potent than **7** (GI_{50} 2.11–6.12 μM).

The impact of the substituent (R^1) at the *para*-position of the phenyl ring (B-ring) in **7** was explored by synthesizing and testing series **9** compounds (**9a**–**k**) for cytotoxic activity. Compared with **9d**, which has an unsubstituted phenyl B-ring (GI_{50} >17 μM), most remaining compounds in this series showed higher potency, indicating that a *para*-substituent on the B-ring might be necessary for cytotoxic activity. Compound **9a** and **9b** with a 4-methoxy or

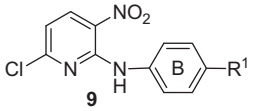


Scheme 1. Synthesis of target compounds **9**–**13**. (a) $NaHCO_3$/EtOH, rt, 12–40 h; (b) microwave irradiation 120–190 °C, K_2CO_3/*t*-BuOH, 10–40 min; (c) K_2CO_3, DMSO, 110 °C, N_2, 4 h; (d) Et_3N for **11a**–**c** or *t*-BuOK for **11d**–**e** in DMF, rt, 30 min; (e) K_2CO_3/*t*-BuOH, rt, 12–24 h.



Scheme 2. Synthesis of target compounds **14a**–**e**. (a) $NaBH_4$, $NiCl_2{\cdot}6H_2O$/MeOH, 0–45 °C, 52%; (b) **14b**: $H_2SO_4/NaNO_2$, CuO_2/EtOH, 50 °C; **14c**: HCl/$NaNO_2$, CuCl/HCl, 50 °C; **14d**: 48%HBr/$NaNO_2$, CuBr/HBr, 80 °C; **14e**: $H_2SO_4/NaNO_2$, KI, $NaHSO_3$, 50 °C; (c) CuCN, $Pd(PPh_3)_4$, I_2/DMF, N_2, 100 °C, 10 h.

Table 1
Inhibitory activity of **9a**–**n** against HTCL panel



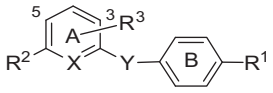
Compd	R^1	GI_{50}[a] (μM)			
		A549	KB	KB-vin	DU145
9a	OMe	13.5	7.19	2.40	3.83
9b	OEt	15.8	3.26	2.26	2.94
9c	Me	21.6	18.1	13.9	15.9
9d	H	23.8	20.8	17.7	20.1
9e	F	21.2	9.57	5.88	11.9
9f	Cl	20.4	15.5	14.7	16.2
9g	I	27.2	29.3	23.2	46.6
9h	OCF_3	9.98	8.03	9.05	13.1
9i	$CHMe_2$	1.47	13.9	13.0	14.7
9j	COOH	19.6	29.1	27.4	25.3
9k	COOMe	9.07	10.2	10.5	4.16
7	CN	6.12	2.73	2.11	3.53
Paclitaxel[b]		0.00489	0.00265	0.948	0.00255

[a] GI_{50} is concentration that inhibits 50% human tumor cell growth, and the values were averaged from at least three independent experiments.
[b] Positive control.

4-ethoxy substituent, respectively, on the B-ring showed similar potency to **7** against KB (not **9a**), KB-vin, and DU145, and both compounds were more potent against drug-resistant KB-vin (GI_{50} 2.40 and 2.26 μM) than KB (GI_{50} 7.19 and 3.26 μM) cells. However, compounds substituted with halogens (F, Cl, I, OCF_3) or carboxylic acid (COOH) or ester groups (COOMe) were less active than **7** against all tested tumor cell lines, see **9e**–**h** and **9j**–**k**. Thus, *para*-cyano (**7**) and *para*-alkoxy substituents on the B-ring might favor enhanced cytotoxic activity. Compounds with *para*-alkyl substituents (**9c**, methyl; **9i**, isopropyl) were generally inactive; however, interestingly, **9i** exhibited significant and selective inhibition of A549 cell growth (GI_{50} 1.47 μM).

Next, modification of the A-ring was explored (Table 2). While **9a** (R^2 = 6-Cl, R^3 = 3-NO_2) was active, **10a** (R^2 = H, R^3 = 3-NO_2), which lacks the 6-chloro (*meta*-chloro) group on the A-ring, was inactive, suggesting that this group is a fundamental cytotoxic pharmacophore. Furthermore, both **10b** (5-NO_2) and **10c** (3,5-NO_2) were also inactive. The next modification involved replacing the pyridine (A-ring) by a benzene ring to produce a series of diphenylamine compounds (**11a**–**11e**). These analogs have a common 6-chloro-3,5-dinitrophenyl moiety (A-ring) and different substituents on the B-ring. Similarly to the series **9** compounds, *p*-methyl substituted analog **11c** showed the lowest potency, while analog **11e** (*p*-cyanophenyl) showed broad cytotoxicity against the HTCL panel (GI_{50} 0.33–3.45 μM). In addition, the four more active series **11** compounds showed different cell line profiles. Analogue **11a** (*para*-OMe) was especially potent against DU145 (GI_{50} 0.93 μM), **11b** (*para*-Cl) was six-fold more potent against KB and KB-vin (GI_{50} 1.37 and 1.34 μM, respectively) than A549 and DU145 (GI_{50} 8.32 and 8.29 μM), **11d** (*para*-NO_2) was three- to eight-fold more potent against A549 (GI_{50} 2.57 μM) than the remaining tumor cell lines, and **11e** (*para*-CN) was particularly potent against drug-resistant KB-vin cell growth (GI_{50} 0.33 μM). Therefore, the *para*-group on the B-ring affected the selectivity against different cell lines.

Table 2
Inhibitory activity of **10**–**14** against HTCL panel



Compd	B-ring	Linker	A-ring			GI_{50}[a] (μM)			
	R^1	Y	X	R^2	R^3	A549	KB	KBvin	DU145
10a	OMe	NH	N	H	3-NO_2	NA[b]	NA	NA	NA
10b	OMe	NH	N	H	5-NO_2	NA	NA	NA	NA
10c	OMe	NH	N	H	3,5-NO_2	NA	NA	NA	NA
11a	OMe	NH	CH	Cl	3,5-NO_2	10.7	3.44	5.16	0.93
11b	Cl	NH	CH	Cl	3,5-NO_2	8.32	1.37	1.34	8.29
11c	Me	NH	CH	Cl	3,5-NO_2	31.1	5.92	6.24	8.84
11d	NO_2	NH	CH	Cl	3,5-NO_2	2.57	19.7	16.6	8.47
11e	CN	NH	CH	Cl	3,5-NO_2	3.45	2.42	0.33	1.07
12	CN	O	CH	Cl	3,5-NO_2	3.07	1.83	1.53	0.84
13a	CN	O	N	Cl	3-NO_2	1.02	1.05	1.81	0.94
13b	OMe	O	N	Cl	3-NO_2	2.00	1.50	1.18	2.03
13c	OEt	O	N	Cl	3-NO_2	10.0	8.31	10.5	8.35
13d	$CHMe_2$	O	N	Cl	3-NO_2	NA	NA	11.6	11.4
13e	COOMe	O	N	Cl	3-NO_2	9.17	8.29	8.55	7.97
14b	OMe	O	N	Cl	3-H	NA	NA	NA	NA
14c	OMe	O	N	Cl	3-Cl	NA	22.7	17.4	NA
14d	OMe	O	N	Cl	3-Br	NA	NA	NA	NA
14e	OMe	O	N	Cl	3-I	NA	NA	NA	NA
14f	OMe	O	N	Cl	3-CN	NA	12.4	12.7	NA
7	CN	NH	N	Cl	3-NO_2	6.12	2.73	2.11	3.53
Paclitaxel[c]						0.00489	0.00265	0.948	0.00255

[a] GI_{50} is concentration that inhibits 50% human tumor cell growth, and the values were averaged from at least three independent experiments.
[b] NA: no activity.
[c] Positive control.

Next, the NH linker was replaced by an isosteric oxygen atom (O) to obtain diarylethers **12** and **13a**–**e**. Compounds **12**, **13a**, and **13b** with a *para*-cyano or -methoxy group on the B-ring exhibited improved potency with GI_{50} values of 0.84–3.07 μM, which were similar to those for **11e**. All three diarylether compounds were more potent than **7** against the HTCL panel. Similarly to the series **9** diarylamines, the diarylether compounds **13d** and **13e** with *para*-isopropyl and *para*-carboxylate substituent in the B-ring were less potent than **13a** and **13b** with *para*-cyano and *para*-methoxy groups. However, **13c** with a *para*-ethoxy group was also significantly less potent than **13b**, unlike the related **9b** and **9a**. Finally, the 3-nitro (*ortho*-nitro) group of active compound **13b** was systemically replaced with H, Cl, Br, I, or CN to give corresponding compounds **14b**–**14f**, resulting in great decreased or abolished antitumor activity against all cell lines (Table 2). Thus, the *ortho*-nitro group on the A-ring might be necessary for optimal activity or interaction with the biologic target.

Subsequently, active compounds **11e**, **12**, **13a**, and **13b** were evaluated against 72 kinases in a high throughput screening platform[16] to explore potential drug target(s). Notably, compounds

Table 3
Inhibitory activity against TAM family of receptor tyrosine kinases

Compd	IC_{50}[a] (μM) in kinase assays			GI_{50} (μM) in K562 cells[b]
	Mer	Tyro-3	Axl	
11e	2.2 ± 1.8	>30	>10	3.72 ± 0.34
12	2.4 ± 1.6	>30	>30	3.01 ± 0.23
13b	3.0 ± 0.5	>30	>10	4.79 ± 0.47
1 (Gleevec)[c]	—	—	—	0.39 ± 0.12

[a] IC_{50} is concentration that inhibits 50% kinase.
[b] GI_{50} is concentration that inhibits 50% human chronic myelogenous leukemia (K562) cell growth by MTS method.
[c] Positive control in the cellular assay. All values were averaged from at least two or more independent experiments.

11e, **12**, and **13b** exhibited inhibitory activity for Mer tyrosine kinase (Mer TK) with IC_{50} values ranging from 2.2–3.0 μM, but did not inhibit Tyro3 and Axl (IC_{50} >30), two other members of the TAM kinase family (Table 3). Because Mer TK was recently identified as a specific therapeutic target for acute lymphoblastic leukemia (ALL),[17] compounds **11e**, **12**, and **13b** were further tested against the K562 (human chronic myelogenous leukemia cell line) using the[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazoliuinm, inner salt] (MTS) colorimetric method[18] in parallel with **1** (gleevec) as the positive control. As expected, the three new compounds did inhibit K562 cell growth with GI_{50} values ranging from 3.01–4.79 μM (Table 3), although they were less potent than **1** (0.39 μM) in the same assay. These results indicated that Mer kinase might be a major target, but not the only target, for these leads, because they were more potent in the cellular assay than the kinase inhibitory assay. However, the current results have provided us with a new starting point to develop selective Mer kinase inhibitors by lead optimization. A Mer TK-selective inhibitor would be a promising option for treatment of pediatric acute lymphocytic leukemia (ALL), other leukemias, and possibly adult solid tumors, because Mer TK is ectopically over-expressed at high levels in pediatric T- and B-cell ALL, but not normal, lymphocytes,[19,20] and also is associated with the pathophysiology of tumorigenesis and thrombosis.

In conclusion, the modifications on hit 7 led to the discovery of new diarylamine and diarylether leads, **11e**, **12** and **13b**, which showed significant inhibitory activity against a human tumor cell line panel (A549, KB, KBvin, Du145, and K652) with low micromolar GI_{50} values ranging from 0.33–4.79 μM, and also inhibited Mer tyrosine kinase with IC_{50} values of 2.2–3.0 μM. The current SAR results suggested that a *meta*-chloro- and *ortho*-nitro group on the A-ring (pyridine or benzene) and a *para*-cyano or methoxy group on the B-ring (see **7**, **11e**, **12** and **13a**, **9a**, **11a**, respectively) are essential pharmacophores for cytotoxic acitivity. Meanwhile, the *para*-substituent on the B-ring and the linker between aryl rings are changeable and appear to modulate the inhibitory selectivity. These promising results provide a good starting point to further optimize the new leads based on the diarylamine or diarylether structural core for developing a novel class of anticancer drugs targeting Mer kinase.

Acknowledgments

This investigation was supported by Grants 81120108022 and 30930106 from the Natural Science Fundation of China (NSFC), and 2006DFA33560 from the Ministry of Science and Technology Commission in China awarded to Lan Xie and NIH Grant CA17625-32 from the National Cancer Institute awarded to K.H. Lee. This study was also supported in part by the Taiwan Department of Health, China Medical University Hospital Cancer Research Center of Excellence (DOH100-TD-C-111-005).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.08.014. These data include MOL files and InChiKeys of the most important compounds described in this article.

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6. *Synthetic Method A*: General procedure for the preparation of **9a**–**9e**. A mixture of **8a** (1.0 mmol), aniline, or substituted aniline (1.0 mmol), and sodium bicarbonate (1.0 mmol) in absolute EtOH (10 mL) was stirred at room temperature for 12–40 h monitored by TLC until the reaction finished. The mixture was poured into ice-water, pH was adjusted to 3.0 with aq HCl (2 N), and solid crude product was collected. The corresponding products were then purified by column chromatograph, PTLC, or re-crystallization.
7. *Synthetic method B (microwave irradiation)*: General procedure for the synthesis of **9f**–**9g**, **9i**–**9k**, **10a**, **10c**, **12**, and **13e**. A mixture of **8a**, **8b**, **8d**, or **8e** (1.0 mmol), a substituted aniline (1.0–1.5 mmol), anhydrous potassium carbonate (2.0 mmol) in 4 mL of *t*-BuOH (except for **12** in DMSO), was heated at 120–180 °C with microwave-assistance for 15–40 min with stirring. After the reaction was finished, the mixture was poured into ice-water, pH was adjusted to ~3.0 with aq HCl (2 N) or to ~10.0 with aq NaOH (5%), and solid crude product was filtered and then purified by column chromatograph, PTLC, or recrystallization to give pure product.
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10. *Synthetic Method D*: Preparations of **11a**–**c**, Et_3N (2 mmol) was slowly added into the solution of **8e** (1.0 mmol) and 4-substituted aniline (1.1 mmol) in DMF (3 mL) at below 0 °C and then was stirred at room temperature for 30 min monitored by TLC. However, the syntheses of **11d**–**e** were performed in the presence of *t*-BuOK (2.0 mmol), rather than Et_3N. After the reaction was finished, the mixture was poured into ice-water, pH was adjusted to 6, and solid was precipitated. The collected crude product was then recrystallized from EtOH to afford corresponding pure products.
11. *Synthetic Method E*: General procedure for the synthesis of the compounds **9h**, **13a**–**13d**. A mixture of **8a** (1.0 mmol), substituted aniline/phenol (1.0–1.5 mmol), and anhydrous potassium carbonate (2 mmol) in *t*-BuOH was stirred at room temperature for 12–24 h monitored by TLC until the reaction completed. When the reaction finished, the mixture was poured into ice-water, pH was adjusted to about 3.0 or 10.0 with aq HCl (2 N) or NaOH (5%), respectively, and the mixture was then extracted with CH2Cl2 (20 mL × 3). After removal of solvent, the crude product was purified by column chromatography, PTLC, or recrystallization to give the corresponding pure products.
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13. *The most active new compounds:* ***11e***: Yellow solid, mp 140–141 °C. ^{1}H NMR δ ppm 7.41 (1H, s, ArH-6), 7.57 (2H, d, J = 8.8 Hz, ArH-2′,6′), 7.91 (2H, d, J = 8.8 Hz, ArH-3′,5′), 8.90 (1H, s, ArH-3), 10.07 (1H, s, NH); MS m/z (%) 319 (M+1+, 100), 321 (M+3+, 23); HPLC purity 100.0%. **12**: Pale yellow solid, mp 118–120 °C. ^{1}H NMR (DMSO-d_6) δ 5.98 (1H, s, ArH-6), 7.12 (2H, d, J = 8.8 Hz, ArH-2′,6′), 7.80 (2H, d, J = 8.8 Hz, ArH-3′,5′), 8.73 (1H, s, ArH-3); MS m/z (%) 318 (M−1, 100), 310 (M+1, 27); HPLC purity 99.51%. **13a**: Pale yellow solid, mp 128–130 °C. ^{1}H NMR δ ppm 7.25 (1H, d, J = 8.4 Hz, PyH-5), 7.34 (2H, d, J = 8.8 Hz, ArH-2′,6′), 7.77 (2H, d, J = 8.8 Hz, ArH-3′,5′), 8.41 (1H, d, J = 8.4 Hz, PyH-4); MS m/z (%) 276 (M+H+, 100), 278 (M+3+, 33); HPLC purity 99.14%. **13b**: Pale yellow solid, mp 110 °C. ^{1}H NMR δ ppm 3.83 (3H, s, OCH3), 6.93 (2H, d, J = 9.2 Hz, ArH-3′,5′), 7.10 (2H, d, J = 9.2 Hz, ArH-2′,6′), 7.11 (1H, d, J = 8.4 Hz, PyH-5), 8.33 (1H, d, J = 8.4 Hz, PyH-4); MS m/z (%) 281 (M+H+, 100), 283 (M+3+, 32); HPLC purity 100.0%.
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18. *MTS assay*: K562 cells (ATCC no. CCL243) are human myelogenous leukaemia, obtained from Lineberger Cancer Center (UNC-CH). Cells were cultured in RPMI 1640 medium containing 25 mM HEPES and 2 mM L-glutamine(Mediatech), supplemented with 10% fetal bovine serum (Hyclone), 100 IU penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Mediatech). Cells were maintained at 37 °C in a humidified 5% CO2 atmosphere. K562 cell viability was determined using a colorimetric assay, Cell Titer 96R AQueous Non-radioactive Cell Proliferation Assay (Promega, Medison, WI) according to the manufacturer's protocol. In brief, the cells (5 × 103 cells/well) were seeded in 96-well plates filled with culture medium containing various concentrations of samples. After 72 h of incubation, 20 μL/well of combined MTS/PMS solution was added and incubated for 2–3 h. The absorbance was measured at 490 nm subtracting the background at 630 nm using a Microplate Reader ELx800 (Bio-Tek Instruments, Winooski, VT) with a Gen5 software. The number of viable cells was counted in a hemocytometer chamber using trypan blue dye exclusion (Sigma). All data represent at least three independent experiments performed in duplicate.
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