Bioorganic & Medicinal Chemistry Letters 23 (2013) 569–573

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Discovery of 4-phenyl-2-phenylaminopyridine based TNIK inhibitors

Koc-Kan Ho *, K. Mark Parnell, Yi Yuan, Yong Xu, Steven G. Kultgen, Steven Hamblin, Thomas F. Hendrickson, Bai Luo, Jason M. Foulks, Michael V. McCullar, Steven B. Kanner

Astex Pharmaceuticals, Inc., 2401 South Foothill Drive, Salt Lake City, UT 84109, USA

ARTICLE INFO

Article history:
Received 28 June 2012
Revised 29 October 2012
Accepted 7 November 2012
Available online 16 November 2012

Keywords:
TNIK
Wnt signaling
β-catenin
TCF4
Phenylaminopyridine

ABSTRACT

A series of compounds based on a 4-phenyl-2-phenylaminopyridine scaffold that are potent and selective inhibitors of Traf2- and Nck-interacting kinase (TNIK) activity are described. These compounds were used as tools to test the importance of TNIK kinase activity in signaling and proliferation in Wnt-activated colorectal cancer cells. The results indicate that pharmacological inhibition of TNIK kinase activity has minimal effects on either Wnt/TCF4/β-catenin-driven transcription or viability. The findings suggest that the kinase activity of TNIK may be less important to Wnt signaling than other aspects of TNIK function, such as its putative role in stabilizing the TCF4/β-catenin transcriptional complex.

The Wnt signaling pathway plays a major role in colorectal cancer. In nearly 90% of colorectal cancers, activating mutations in Wnt pathway components lead to increased transcription through the TCF4/β-catenin transcriptional complex, resulting in increased cell proliferation.[1–3] Recent reports have identified Traf2- and Nck-interacting kinase (TNIK) as critical for Wnt signaling and proliferation of colon cancer cells,[4,5] and small molecule inhibitors were reported.[6] Evidence suggests that in response to Wnt signaling, TNIK localizes to the nucleus and phosphorylates TCF4 on Ser154, and that the kinase activity of TNIK is necessary for TCF4 transcriptional activity and maintenance of colorectal cancer growth.[5] These findings suggest that small molecule TNIK inhibitors may be useful therapeutic agents in colorectal cancers with aberrant Wnt signaling.

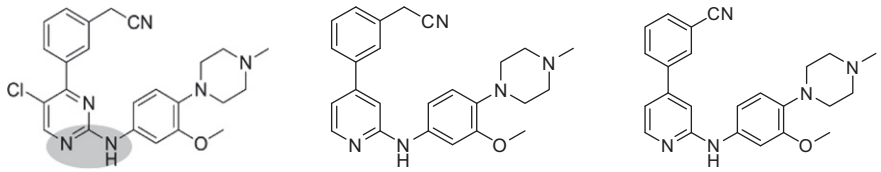
A biochemical assay was developed to screen for TNIK inhibitors utilizing the Promega ADP Glo system, in which ADP formed during the kinase reaction produces a proportional luminescent signal.[7] Using this assay, a small in-house collection of compounds (~500) with known or predicted kinase inhibitory activity were screened. Among the screen hits was compound **1** which inhibited TNIK biochemical activity with an IC_{50} = 65 nM (Table 1). Compound **1** also inhibited the binding of ATP to TNIK in an orthogonal ATP competition assay (K_d = 86 nM, K_dELECT, KINOMEscan, DiscoveRx, CA, USA).

To determine the kinase selectivity of **1**, the compound was screened against a panel of 118 oncogenic kinases (oncoKP, Reaction Biology, PA, USA). More than half of the kinases in the panel were inhibited by at least 50% at 1 μM concentration of **1** (*S*(50) = 56%, Table 2), indicating poor selectivity. This was not a surprising result given that the phenylaminopyrimidine moiety in **1** is a common motif found in many kinase inhibitors. Consequently, a tool compound with significant improvement in general kinase selectivity was needed in order to interrogate the target. One of the approaches to improve kinase selectivity was to alter the nature of the hinge binding domain, therefore the aminopyrimidine moiety in compound **1** (Table 1). Replacing the pyrimidine ring in **1** with a pyridine yielded a structurally related compound **2**, in which the calculated pK_a (ACD/pK_a, Advanced Chemistry Development, Inc., Ontario, Canada) of the pyridine nitrogen is 5.08 as compared with 0.18 for the corresponding pyrimidine nitrogen. It was postulated that altering the pK_a could change the property of the hinge binding interactions and could change the selectivity profile of a compound. Although compound **2** was less potent against TNIK (IC_{50} = 290 nM, ADP Glo; K_d = 220 nM, K_dELECT), it appeared to be more selective than **1** against a small number of in-house kinase targets (data not shown). This observation led us to explore 4-phenyl-2-phenylaminopyridines as a potential scaffold for TNIK inhibitors. Replacement of the 3-cyanomethyl group in **2** with a 3-cyano (**3**) increased the inhibition against TNIK to IC_{50} of 6 nM. Compound **3** was over 40-fold more potent than the parent compound **2**, and 10-fold more potent than screening hit **1**. Kinase selectivity analysis of **3** showed that only 6 of the 118 kinases in the oncoKP panel were inhibited by 50% or more at 1 μM concentration of **3** (*S*(50) = 5.1, Table 2). In addition, it was found that TNIK was the

* Corresponding author. Tel.: +1 732 896 7363.
E-mail address: kanho99@yahoo.com (K.-K. Ho).

0960-894X/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmcl.2012.11.013

Table 1
Properties of initial TNIK hits



1[a] **2** **3**

	1[a]	**2**	**3**
Molecular weight	449.0	413.5	399.5
TNIK IC_{50} (nM)	65	290	6
Solubility (μM)[b]	Not determined	94	78
Caco-2 $P_{app(A-B)}$ (nm/sec)	80	205	160
Caco-2 $P_{app(B-A)}$ (nm/sec)	77	191	142
Caco-2 $P_{app(B-A)}/P_{app(A-B)}$	0.96	0.93	0.89

[a] Highlighted area represents the hinge binding domain.
[b] Solubility was determine at100 mM phosphate buffer, pH 7.4.

most potently inhibited kinase, with inhibition of >99% at 1 μM concentration. The only other kinase with less than 95% inhibition at 1 μM concentration of **3** was MAP4K4 (98% inhibition at 1 μM concentration), which is 90% identical to TNIK in its kinase domain sequence.[8] It was concluded that the 4-(3-cyanophenyl)-pyridine core of **3** interacts with the TNIK in high selectivity.

To gain insight on the compound's binding poses to the TNIK active site, modeling studies were performed on compounds **1** and **3** using a recently published TNIK crystal structure (PDB entry 2X7F). Figure 1 shows energy minimized poses for **1** and **3**. Both compounds share a common binding motif, with classic 'hinge binding' hydrogen bonds between the aminopyrimidine (**1**) or aminopyridine (**3**) moieties and the hinge region backbone amide NH of Phe107 and the carbonyl O of Cys108. The protonated methylpiperazine group is predicted to interact through a salt bridge with the carboxy group of Asp115. The principal difference in the

Table 2
Activity of compounds **1** and **3** against a panel of oncogenic kinases

	1	**3**
Selectivity score *S*(50)[a]	56%	5.1%
>95% Inhibition at 1 μM	JAK2, VEGFR3, JAK3, RET, FGFR1, FLT3, IKKε, VEGFR2, p70S6K, Aurora A, VEGFR1, ROS, FGFR2, FGFR3, TRKC	TNIK, MAP4K4
95 to >80% Inhibition at 1 μM	ABL2, Aurora B, CDK4/cyclin D1, FMS, MEK1, BRK, CDK6/cyclin D1, CHK1, FER, CHK2, YES, PDGFRβ, LCK, c-SRC, ABL1, NEK1, HIPK3, MEK2, HIPK4, TRKA, Syk, JAK1, AXL, LYN, FES, IGF1R, PDGFRα, MUSK, Aurora C, DAPK1, CK2α, c-KIT, TRKB	IKKε
80–50% Activity at 1 μM	FAK, PAK4, EPHB1, FGR, CDK9/cyclin T1, FYN, PYK2, PKCδ, EPHA1, CDK2/cyclin A, EPHA7, HIPK1, PKCε, PKCθ, MST4, CDK1/cyclin B, HCK, TIE2	FLT3, CDK6/cyclin D1, FMS, CHK1

[a] *S*(50) indicates the percentage of kinases in panel (118 total) inhibited by >50% at 1 μM concentration.

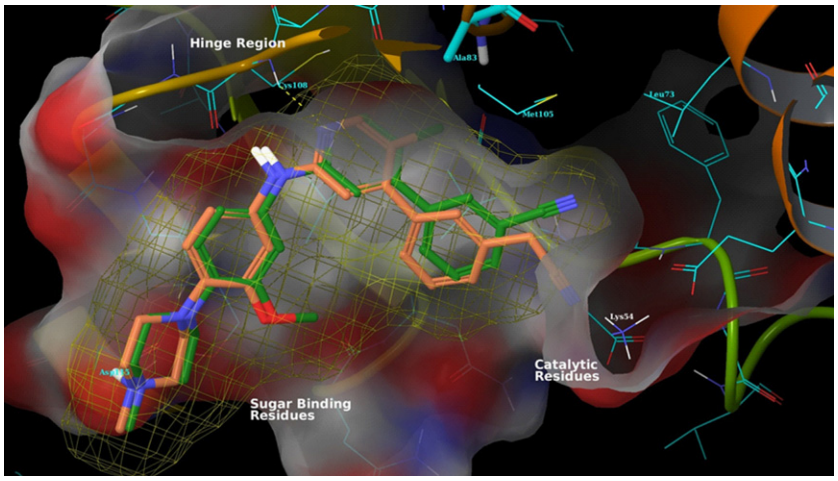
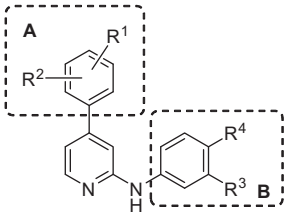
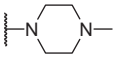
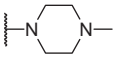
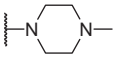
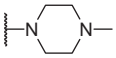
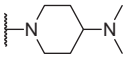
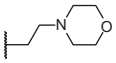


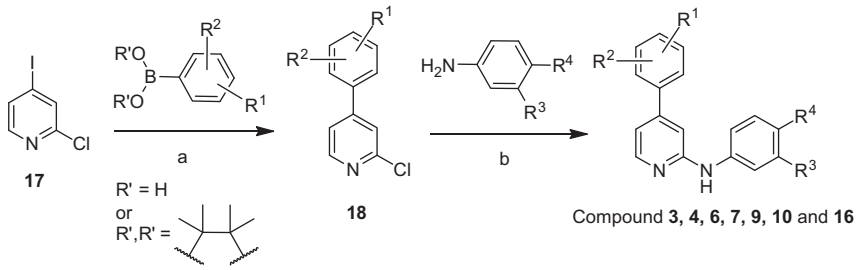
Figure 1. Compounds **1** (brown carbons) and **3** (green carbons) in the ATP binding pocket. The surface was generated from the TNIK protein with several of the flap residues removed for clarity. Hinge region, sugar binding region, and catalytic residues are indicated. The yellow mesh is the molecular surface for compound **3**. The modeling study was performed using a TNIK crystal structure (PDB entry 2X7F). Compounds **1** and **3** were docked to the protein and energy minimized using GLIDE and MacroModel (Schrödinger, NY, USA), respectively.

Table 3
SAR for 4-phenyl-2-phenylaminopyridine based TNIK inhibitors



Compound	R^1	R^2	R^3	R^4	TNIK IC_{50} (nM)	hERG[a] IC_{50} (μM)
3	3-CN	H	MeO		6	1.3
4	3-CN	6-MeO	H		5	
5	3-CN	6-MeO	MeO		3	
6	3-Cl	6-MeO	MeO		4	
7	3-CN	H	MeO		55	7.7
8	3-CN	6-MeO	H		11	
9	3-CN	6-MeO	MeO		8	4.7
10	4-CN	H	MeO		>1,000	
11	3-Cl	6-MeO	MeO		41	20
12	3-F	6-MeO	MeO		118	
13	3-CF3	6-MeO	MeO		275	
14	3-CN	5-MeO	MeO		63	
15	3-CN	6-MeO	F		11	
16	3-Cl	6-MeO	H		20	

[a] hERG IC_{50} was determined by a FastPatch assay (WuXi PharmaTech, Shanghai, PRC).

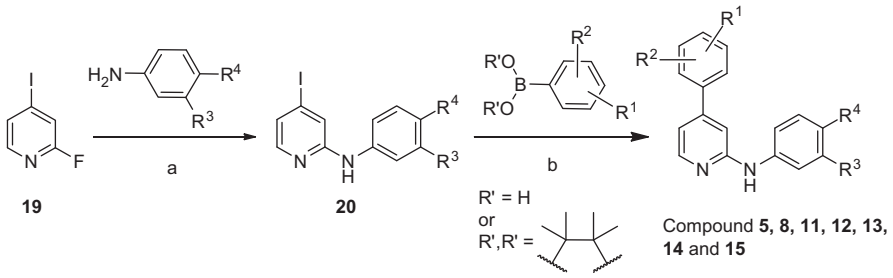


Scheme 1. Reagents and conditions: (a) $Pd(PPh_3)_4$, Na_2CO_3, dioxane/water, 100 °C, 4 h. (b) HCl, *i*PrOH, microwave 170 °C, 12 h.

binding modes of **1** and **3** is in the substituted phenyl group, which in both cases is oriented toward the catalytic residues. In compound **1**, the nitrogen of the cyanomethyl group accepts a hydrogen bond from the catalytic Lys54. In compound **3**, there are no predicted hydrogen bonds, as the cyano group is directly attached to the phenyl ring and is therefore much less flexible. Instead, the cyano group is nestled in a mostly hydrophobic pocket between residues Leu103, Leu73 and Met105, which is an energetically favorable interaction according to our model. The position of the cyano group allows both the pyridine ring and the phenyl ring of compound **3** to interact more closely with residues Met105, Val170 and Ala83, picking up additional hydrophobic interactions.

Compounds **1–3** were tested in a Caco-2 assay to determine cell permeability. Compounds with a pyridine core (**2** and **3**), demonstrated Caco-2 permeability coefficients at 160–205 nm/s that were roughly 2-fold greater than the pyrimidine **1**, with no evidence of efflux (Table 1). Therefore, cell permeability is unlikely the limiting factor for triggering a cellular response for the series.

Given that **3** was potent against TNIK, highly selective, and cell permeable, medicinal chemistry efforts were focused on



Scheme 2. Reagents and conditions: (a) HCl, dioxane/water 102 °C, 20 h. (b) Pd(dppf)Cl_2, K_2CO_3, dioxane/water 80 °C, 16 h.

Table 4
Comparison of biochemical inhibition of TNIK and cell-based inhibition of Wnt-driven β-lactamase for a set of 4-phenyl-2-phenylaminopyridines

Compound	TNIK IC_{50} (nM)	β-lactamase IC_{50} (nM)	β-lactamase IC_{50}/TNIK IC_{50}
3	6	27,000	4500
4	5	>30,000	>6000
6	4	7900	2000
8	11	26,000	2400
9	8	6300	790
15	11	3100	280
1	65	350	5.4
Staurosporine	1.4	39	28

eliminating the hERG liability associated with the highly basic methylpiperidinyl moiety in compound **3**. Through patch clamp analysis, it was determined that **3** inhibited hERG with an IC_{50} of 1.3 μM (Table 3). However, given the potential of hERG-based clinical cardiac toxicity,[9] our goal was to reduce the potency of the series against the hERG channel.

A series of analogs (Table 3) were prepared by a two step synthesis utilizing a Suzuki coupling and a S_NAr reaction. Using 2-chloro-4-iodopyridine **17** as starting material, a boronic acid or ester was selectively coupled to the 4 position of the pyridine (Scheme 1). The resulting chloropyridine **18** was then reacted with an aniline to yield the desired analogs. The reaction sequence can be reversed when a starting material of 2-fluoro-4-iodopyridine **19** was used (Scheme 2).

Replacement of the 4-methylpiperazine of **3** with a non-basic morpholine as in compound **7** decreased activity against both hERG (IC_{50} = 8 μM) and TNIK (IC_{50} = 55 nM) (Table 3). Substitution of a 6-methoxy in the R^2 (compound **9**) increased TNIK activity (IC_{50} = 8 nM) while only modestly increasing hERG activity (IC_{50} = 4.7 μM) as compared with **7**. Further reduction in hERG activity was achieved by replacing the cyano group in R^1 with a chloro moiety yielding compound **11** with hERG IC_{50} of 20 μM.

Initial SAR suggested that a methoxy substitution either in the 6-position of R^2 or in R^3 does not affect the potency when the R^4 is a methylpiperidine. The methylpiperidine analogs **3, 4, 5** and **6** show similar potency. However, a more significant improvement in potency is observed for the di-methoxy analog **9** as compared with the mono-methoxy analog **7** when R^4 is a morpholine. For Ring A, it is observed that a 3-cyano is preferred over 4-cyano group in R^1 since compound **10** is devoid of TNIK activity. The 3-cyano in the A ring can also be replaced with a 3-chloro group (**11**), but inferior activity was observed for a 3-fluoro (**12**) or a trifluoromethyl (**13**) compounds. Comparing the activity of compounds **9** and **14**, it is evident that 6-methoxy is preferred over the 5-methoxy moiety. In addition, compounds **15** and **16** with modifications in Ring B were prepared to expand the chemical diversity, so multiple chemotypes could be evaluated for cellular activity.

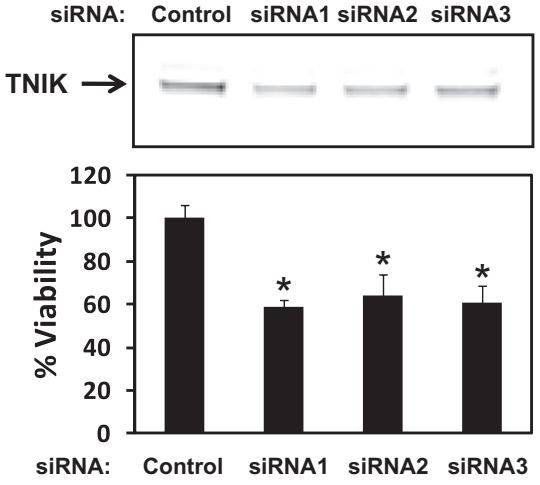


Figure 2. RNAi knockdown of TNIK in HCT-116 cells correlates with a decrease in viability. Top panel indicates TNIK levels, measured by Western blot with anti-TNIK antibody, following transient transfection with siRNAs 1 through 3 targeting TNIK, as well as scrambled siRNA control. Bottom panel indicates% viability of siRNA transfected HCT-116 cells. Control siRNA was purchased from Thermo Fisher Scientific (Non-targeting siRNA D-001810-01-05). TNIK siRNA was purchased from Qiagen (Cat # SI02225503, SI03649856, and SI05187980). *The *P*-value for each siRNA versus control was <0.01.

Having developed a series of potent and selective TNIK inhibitors, the next step was to explore the importance of TNIK kinase activity in Wnt-activated colorectal cancer cells. The hypothesis, based on previous studies was that pharmacological inhibition of TNIK activity would block TCF4/β-catenin-dependent transcription.[4,5] To test this hypothesis, a Wnt-activated colorectal cancer cell line (HCT-116) with a stably expressed TCF4/β-catenin-response element upstream of a β-lactamase reporter gene (CellSensor®, Invitrogen)[10] was used. In short, Wnt signaling through TCF4/β-catenin transcription results in constitutive expression of β-lactamase, and the activity of β-lactamase is measured by conversion of its substrate into a product with a shifted fluorescent signal. Several TNIK inhibitors were tested in this assay and the results are summarized in Table 4. The IC_{50} values for the compounds tested in these cells ranged from 3.1 to >30 μM, more than 280-fold higher than the biochemical TNIK IC_{50} values, and much higher than expected if TNIK kinase activity were essential for TCF4/β-catenin transcription. ICG-001, a compound that prevents the interaction of β-catenin with the co-activator CBP and thus inhibits TCF4/β-catenin-dependent transcription, was included as a positive control.[11] We observed an IC_{50} of 1.3 μM for ICG-001, identical to the published IC_{50} of 1.3 μM,[11] indicating that the assay was indeed responsive to signaling through the TCF4/β-catenin pathway. We found that analog **1**, which was observed to be a non-selective kinase inhibitor, inhibited the β-lactamase reporter assay with an

Table 5
Comparison of biochemical inhibition of TNIK and viability inhibition for Wnt-active and Wnt-inactive cell lines

Compound	TNIK IC_{50} (nM)	Viability IC_{50} (nM)			
		Wnt-active		Wnt-inactive	
		HCT-116	DLD1	HEK-293	HeLa
9	8	2300	5800	105	6800
15	11	260	330	43	1200
16	20	1700	1100	59	3700

IC_{50} of 350 nM. Similarly, staurosporine demonstrated an IC_{50} of 39 nM in the same assay. These results suggested that Wnt signaling through TCF4/β-catenin is sensitive to kinase signaling, but the kinase activity of TNIK is nonessential for that function.

The viability of colorectal cells with activated Wnt signaling has been shown to be TNIK-dependent through RNAi-based knockdown experiments,[4,5] though the effect on viability was not shown to be dependent upon TNIK kinase activity. In our studies, RNAi-based knockdown of TNIK resulted in a corresponding decrease in viability in HCT-116 cells (Fig. 2), but not in Wnt-inactive HeLa cells (data not shown). In contrast, the viability effects observed with TNIK inhibitors were not specific to Wnt-active colorectal cancer cells (Table 5). Indeed, the compounds appeared most toxic to Wnt-inactive HEK-293 cells. The data suggests that viability effects by the TNIK inhibitors (such as **15**) were likely due to effects on pathways other than the Wnt signaling pathway.

In conclusion, a series of potent and selective small molecule TNIK inhibitors were identified. Starting with **1**, a relatively promiscuous compound, potency and selectivity were subsequently built in with few modifications. These tool compounds were used for target validation. Here, pharmacological inhibition of TNIK did not inhibit Wnt signaling through TCF4/β-catenin-mediated transcription, whereas less selective kinase inhibitors, such as compound **1** and staurosporine, inhibited Wnt signaling (Table 4). It remains unlikely that this result was due to compound impermeability, as Caco-2 data indicates that pyridine-containing compounds such as **3** were cell permeable (Table 1). The more likely explanation is that TNIK kinase activity may not be necessary for Wnt signaling, and that **1** and staurosporine inhibit Wnt signaling through inhibition of a kinase(s) unrelated to TNIK. Consistent with a lack of dependency of TNIK kinase activity in Wnt-driven transcription, a Wnt pathway specific viability effect was not observed with the TNIK inhibitors (Table 5). In contrast, a viability effect in Wnt-activated cells upon siRNA knockdown of TNIK was observed (Fig. 2). These results indicate that TNIK protein (though not TNIK kinase activity) is important for viability in Wnt-activated colorectal cancer cells. Given that TNIK has been described as an interacting partner of Traf2,[12] Nck,[12] and TCF4,[5] it remains possible that scaffolding interactions may play an important role in its function in Wnt-activated colorectal cancer cells.

In addition to the proposed role for TNIK in Wnt signaling, TNIK has been implicated in actin cytoskeleton regulation,[8] as a mediator of Ras family member Rep2, which regulates cell proliferation, differentiation, and cytoskeletal rearrangement.[13] Recent evidence has also identified TNIK as a schizophrenia susceptibility gene, influencing neural circuit synchrony.[14] The above-described TNIK inhibitors may be useful tools to interrogate these aspects of TNIK biology.

The compounds described here also inhibit the closely-related MAP4K4 kinase (Table 2),[8] which has been implicated in hepatoma carcinoma growth[15] and invasion,[16] as well as a general factor promoting tumor cell motility.[17,18] MAP4K4 expression is correlated with metastasis and inversely correlated with survival in colorectal cancer,[19] and is associated with poor prognosis in pancreatic ductal adenocarcinoma.[20] Thus, these inhibitors may have utility to better understand the function of MAP4K4 in cancer.

References and notes

1. Morin, P. J.; Sparks, A. B.; Korinek, V.; Barker, N.; Clevers, H.; Vogelstein, B.; Kinzler, K. W. *Science* **1997**, *275*, 1787.
2. Kinzler, K. W.; Vogelstein, B. *Cell* **1996**, *87*, 159.
3. Sparks, A. B.; Morin, P. J.; Vogelstein, B.; Kinzler, K. W. *Cancer Res.* **1998**, *58*, 1130.
4. Mahmoudi, T.; Li, V. S.; Ng, S. S.; Taouatas, N.; Vries, R. G.; Mohammed, S.; Heck, A. J.; Clevers, H. *EMBO J.* **2009**, *28*, 3329.
5. Shitashige, M.; Satow, R.; Jigami, T.; Aoki, K.; Honda, K.; Shibata, T.; Ono, M.; Hirohashi, S.; Yamada, T. *Cancer Res.* **2010**, *70*, 5024.
6. Yamada, T.; Shtashige, M.; Yokota, K.; Sawa, M.; Morityama, H. WO2010/064111, International Publication Date 10, June 2010.
7. Zegzouti, H.; Zdanovskaia, M.; Hsiao, K.; Goueli, S. A. *Assay Drug Dev. Technol.* **2009**, *7*, 560.
8. Taira, K.; Umikawa, M.; Takei, K.; Myagmar, B. E.; Shinzato, M.; Machida, N.; Uezato, H.; Nonaka, S.; Kariya, K. *J. Biol. Chem.* **2004**, *279*, 49488.
9. Sanguinetti, M. C.; Tristani-Firouzi, M. *Nature* **2006**, *440*, 463.
10. Hancock, M. K.; Lebakken, C. S.; Wang, J.; Bi, K. *Mol. Biosyst.* **2010**, *6*, 1834.
11. Emami, K. H.; Nguyen, C.; Ma, H.; Kim, D. H.; Jeong, K. W.; Eguchi, M.; Moon, R. T.; Teo, J. L.; Kim, H. Y.; Moon, S. H.; Ha, J. R.; Kahn, M. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 12682.
12. Fu, C. A.; Shen, M.; Huang, B. C.; Lasaga, J.; Payan, D. G.; Luo, Y. *J. Biol. Chem.* **1999**, *274*, 30729.
13. Bos, J. L. *Biochim. Biophys. Acta* **1997**, *1333*, M19.
14. MacLaren, E. J.; Charlesworth, P.; Coba, M. P.; Grant, S. G. *Mol. Cell. Neurosci.* **2011**, *47*, 93.
15. Liu, A. W.; Cai, J.; Zhao, X. L.; Jiang, T. H.; He, T. F.; Fu, H. Q.; Zhu, M. H.; Zhang, S. H. *Clin. Cancer Res.* **2011**, *17*, 710.
16. Han, S. X.; Zhu, Q.; Ma, J. L.; Zhao, J.; Huang, C.; Jia, X.; Zhang, D. *World J. Gastroenterol.* **2010**, *16*, 4541.
17. Collins, C. S.; Hong, J.; Sapinoso, L.; Zhou, Y.; Liu, Z.; Micklash, K.; Schultz, P. G.; Hampton, G. M. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 3775.
18. Wright, J. H.; Wang, X.; Manning, G.; LaMere, B. J.; Le, P.; Zhu, S.; Khatry, D.; Flanagan, P. M.; Buckley, S. D.; Whyte, D. B.; Howlett, A. R.; Bischoff, J. R.; Lipson, K. E.; Jallal, B. *Mol. Cell. Biol.* **2003**, *23*, 2068.
19. Hao, J. M.; Chen, J. Z.; Sui, H. M.; Si-Ma, X. Q.; Li, G. Q.; Liu, C.; Li, J. L.; Ding, Y. Q.; Li, J. M. *J. Pathol.* **2010**, *220*, 475.
20. Liang, J. J.; Wang, H.; Rashid, A.; Tan, T. H.; Hwang, R. F.; Hamilton, S. R.; Abbruzzese, J. L.; Evans, D. B. *Clin. Cancer Res.* **2008**, *14*, 7043.