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Synthesis and RET protein kinase inhibitory activity of 3-arylureidobenzylidene-indolin-2-ones

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Abstract—A novel series of 3-arylureidobenzylidene-indolin-2-ones was synthesized and their inhibitory activity against Ret tyrosine kinase investigated in comparison with the Ret inhibitor RPI-1 as a reference compound for this series. A few compounds were able to revert the RETC634R oncogene-transformed morphologic phenotype of NIH3T3^{MEN2A} cells and showed a selective antiproliferative activity against these cells as compared to parental NIH3T3 cells or NIH3T3 cells transformed with a non-tyrosine kinase oncogene (NIH3T3$^{H\text{-}RAS}$). Inhibition of Ret enzyme activity by effective derivatives was confirmed in a kinase assay. Structure–activity relationship indicated a favourable activity for 5,6-dimethoxyindolinone derivatives with H, OH, or OMe in the *para* position of the distal aryl ring.

The RET protooncogene is a typical example of a gene encoding a receptor protein tyrosine kinase involved in the aetiology of human tumours.[1] Activating RET mutations and rearrangements have been implicated in the development of sporadic and inherited forms of thyroid cancer. In papillary carcinomas, RET oncogenes, designated RET/PTCs, derive from somatic chromosomal rearrangements linking the promoter and N-terminal domains of unrelated genes to the C-terminal fragment of the RET protooncogene. As a result, chimeric, constitutively active, forms of Ret tyrosine kinase are ectopically expressed in thyroid cells.[2,3] In sporadic cases of medullary thyroid carcinoma (MTC), RET missense mutations are present at a somatic level, whereas RET mutations at a germline level are responsible for the inherited type-2 Multiple Neoplasia (MEN2) syndromes which include MTC.[4,5] RET mutants identified in thyroid carcinomas are dominantly acting oncogenes which confer gain-of-function to the encoded proteins. In fact, all RET oncoproteins are characterized by constitutive ligand-independent tyrosine kinase activity that is required for their transforming ability.[6] RET oncogene transforming activity can be assessed by transfection assays on NIH3T3 cells and is typically associated with constitutive tyrosine phosphorylation of Ret oncoproteins, alteration of cell morphology, anchorage-independent growth in vitro and tumorigenicity in vivo.[7]

Different strategies designed to block the oncogenic activity of RET, including gene therapy approaches, monoclonal antibodies, neutralizing aptamers and tyrosine kinase inhibitors, have provided evidence that RET oncogenes and their products are potential targets for selective thyroid cancer therapy.[8,9] However, a clinically useful therapeutic option for treating patients with RET-associated cancers is still not available. Over the past few years, a number of small-molecule tyrosine kinase inhibitors have been shown to inhibit Ret activity. They include natural products such as the fungal metabolites clavilactones[10] and several synthetic compounds including indolocarbazoles (CEP-701, CEP-751),[11,12] pyrazolopyrimidines (PP1, PP2),[13–15] anilinoquinazolines (ZD6474),[16] pyrrolopyrimidines (AEE788),[17] pyridinyloxyphenylureas (BAY 43-9006)[18] and indolin-2-one derivatives (RPI-1[2,19,20] and SU11248[21]).

Keywords: RET; Protein kinase; Antitumour; Thyroid cancer; Indolinone; Synthesis.
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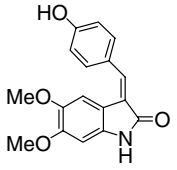
In an effort to improve the Ret inhibitory activity of RPI-1 (Chart 1), we designed a series of new compounds derived by insertion of a diphenyl urea moiety on the active nucleus of 5,6-dimethoxyindolinone. The importance of the diphenyl urea moiety for the inhibition of a number protein kinases, including p38 MAPK, RAF-1 and CDKs, is consistent with earlier observations regarding this structural motif.[22] Recently, other classes of diphenyl urea derivatives have been shown to be potent inhibitors of KDR tyrosine kinase.[23]

Compounds **1**–**11** (Table 1) were synthesized[24] by condensation of the appropriate indolin-2-one with the corresponding 4-arylureidobenzaldehyde in the presence of piperidine. The aldehydes were obtained in turn by reaction of 4-formylphenylisocyanate with the appropriate arylamine (Scheme 1). Compound **12** was obtained by the same sequence but using 4-methylpiperazine. In the case of **13**, the required aldehyde was prepared by coupling 4-carboxybenzaldehyde with aniline.

Compound **14** was obtained by hydrogenation of **3** in the presence of Pd/C (Chart 2). In general, the coupling reaction gave the *E* isomer as the major product, except in the case of compounds **1**, **2** and **10**. Therefore the presence of the 5,6-dimethoxy substitution seems to favour strongly the *E* product. The assigned configuration of the compounds was based upon the chemical shifts of the protons at the C-2′ and C-6′ positions in the phenyl ring of the benzylidene moiety (Table 1). It has been demonstrated through NOE experiments that the chemical shift of C-2′ and C-6′ protons is in the range 7.45–7.84 ppm for the *E* isomers and 7.85–8.53 for the *Z* isomers.[25]



RPI-1

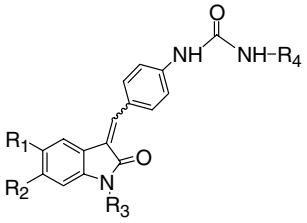
Chart 1.

A cell-based screening[26] was performed to assess inhibition of RET-driven transformation by the synthesized compounds. The inhibition of RET transforming activity was evaluated by using NIH3T3 cells transformed by the C634R mutant of RET associated with the MEN2A syndrome (NIH3T3^{MEN2A}) as a model system.[15] Compounds able to revert the oncogene-transformed morphologic phenotype of NIH3T3^{MEN2A} cells were further tested on NIH3T3 cells and NIH3T3 cells transformed with a non-tyrosine kinase oncogene (NIH3T3$^{H\text{-}RAS}$)[15] to compare the antiproliferative effect of each compound on the three cell lines. Furthermore, the same compounds selected as the most effective were subjected to a Ret kinase assay to assess direct enzyme inhibition. Ret inhibition in NIH3T3^{MEN2A} cells was assessed by the receptor autophosphorylation in Western blot analysis. In each test, RPI-1 [3-(4-hydroxybenzylidene)-5,6-dimethoxyindolin-2-one], previously characterized as a Ret inhibitor,[19] was used as the reference compound.

The results of the cell-based screening and kinase assay with the synthesized compounds are reported in Table 2.

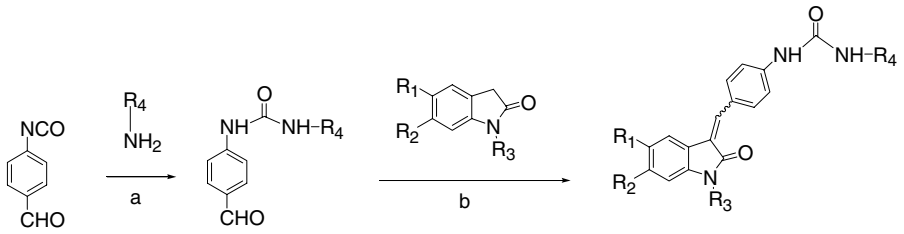
Two compounds in this series (**3** and **4**) induced a stable reversion (up to 72 h) of the RET-induced transformed phenotype of NIH3T3^{MEN2A} cells, with IC_{50} comparable to that of the reference compound RPI-1. Compounds **6** and **7** induced a partial reversion of the transformed cell morphology (cell flattening) associated,

Table 1. Structure of new 3-arylureidobenzylidene-indolin-2-ones



Compound	Config.	R_1	R_2	R_3	R_4	mp (°C)	% Yield	δ[a]
1a	*Z*	H	H	H	Ph	265–267	23	8.44
1b	*E*	H	H	H	Ph	224–226	50	7.61
2	*Z*	Cl	H	H	Ph	271–273	35	8.46
3	*E*	OMe	OMe	H	Ph	255–258	65	7.61
4	*E*	OMe	OMe	Me	Ph	250–252	32	7.61
5	*E*	OMe	OMe	H	4-F-Ph	255–258	65	7.61
6	*E*	OMe	OMe	H	4-OH-Ph	250–252	32	7.61
7	*E*	OMe	OMe	H	4-OMe-Ph	260 (dec)	25	7.60
8	*E*	OMe	OMe	H	4-NMe_2-Ph	248–250 (dec)	58	7.58
9	*E*	OMe	OMe	H	3,4-(OCH_2O)-Ph	>200 (dec)	40	7.62
10	*Z*	H	H	OH	Ph	249–250	52	8.44
11	*E*	OMe	OMe	H	4-Py	264–265	46	7.59

[a] Chemical shift of the 2′ and 6′ protons on the proximal aryl ring.



Scheme 1. Reagents and conditions: (a) Toluene, reflux, 2–5 h; (b) EtOH, piperidine, 90 °C, 3–7 h.

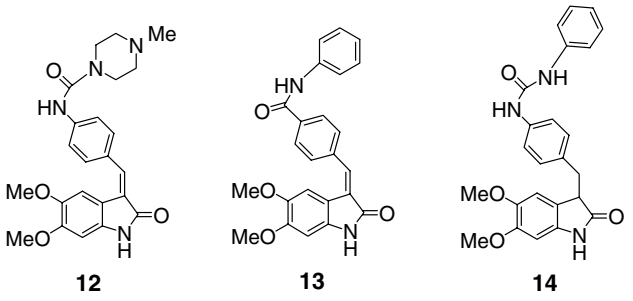


Chart 2.

particularly in the case of **7**, with cell suffering (Fig. 1). Compound **12** induced a transient reversion of the transformed cell phenotype which was lost after 24 h of treatment. Conversely, none of these compounds affected the transformed morphology of NIH3T3$^{H\text{-}RAS}$ cells (Fig. 1).

Similar to RPI-1, compounds **3**, **6** and **7** showed a selective antiproliferative effect against NIH3T3^{MEN2A} cells as compared to that of NIH3T3$^{H\text{-}RAS}$ cells or the parental NIH3T3 cells, thus confirming that RET oncogene expression could be a major determinant of cell sensitivity to drug treatment. Compounds **3**, **4**, **6**, **7** and **12** were tested for their inhibitory activity in a Ret kinase assay[27] in comparison with RPI-1. The results indicated compound **6** as the most effective inhibitor. Comparable IC_{50} values were obtained for RPI-1 and **3**, a lower activity for **6**, while **4** and **12** were inactive or almost inactive, respectively. Compound **4** showed no selectivity among the three cell lines, suggesting a different mechanism of action. Indeed, N-methylation resulted in a loss of ability to inhibit Ret kinase activity as documented for compound **4** and the *N*-methyl derivative of RPI-1 (not shown). The ability of **4** to induce reversion of the transformed phenotype likely reflects modulation of downstream events implicated in various survival pathways. This interpretation is consistent with efficacy of **4** in H-RAS transformed cells and in a partial reversion of these cells (not shown).

Western blot analysis confirmed the ability of compounds **3** and **6** of inhibiting Ret tyrosine kinase in NIH3T3^{MEN2A} cells as indicated by dose-dependent inhibition of the receptor autophosphorylation. In contrast to the cell-free enzyme assay, compound **3** showed higher efficacy than compound **6** in cell culture (Fig. 2). As previously reported for RPI-1,[20] Ret kinase inhibition in treated cells was associated with a reduction of RET protein expression.

The results of this study allow some consideration as to the structure-activity relationship. Comparison of the biochemical and cellular effects of the compounds that proved able to induce the reversion of the transformed phenotype provides the following information:

Table 2. In vitro activity of the synthesized compounds

	NIH3T3^{MEN2A} reversion (10 μM)	Antiproliferative activity (IC_{50} in μM) (72 h)			Ret Kinase Assay[b] (IC_{50}μM)
		NIH3T3^{MEN2A}	NIH3T3	NIH3T3$^{H\text{-}RAS}$	
RPI-1	YES (s)[a]	3.6 ± 1.6	16.3 ± 3.7	19.7 ± 2	0.17 ± 0.01
1a	NO	>40			
1b	NO	10 ± 0.7			
2	NO	>40			
3	YES (s)[a]	4.6 ± 0.1	14 ± 2.2	8.7 ± 0.1	0.2 ± 0.001
4	YES (s)[a]	4.1 ± 1	6.6 ± 0.8	5.4 ± 1.4	Inactive[c]
5	NO	6.1 ± 1.7			
6	YES (p)[a]	8.2 ± 1.8	24.4 ± 0.1	22.2 ± 3	0.05 ± 0.02
7	YES (p)[a]	3.6 ± 1	14.7 ± 2	12.3 ± 1.3	0.58 ± 0.22
8	NO	>40			
9	NO	19.4 ± 2			
10	NO	7.5 ± 0.1			
11	NO	4.7 ± 0.1			
12	YES (t)[a]	15.8 ± 0.7			>3
13	NO	12.2 ± 0.4			
14	NO	21.5 ± 2.8			

[a] (s), stable; (p), partial; (t), transient.
[b] Phosphorylation of MBP as substrate.
[c] inactive up to 3μM.

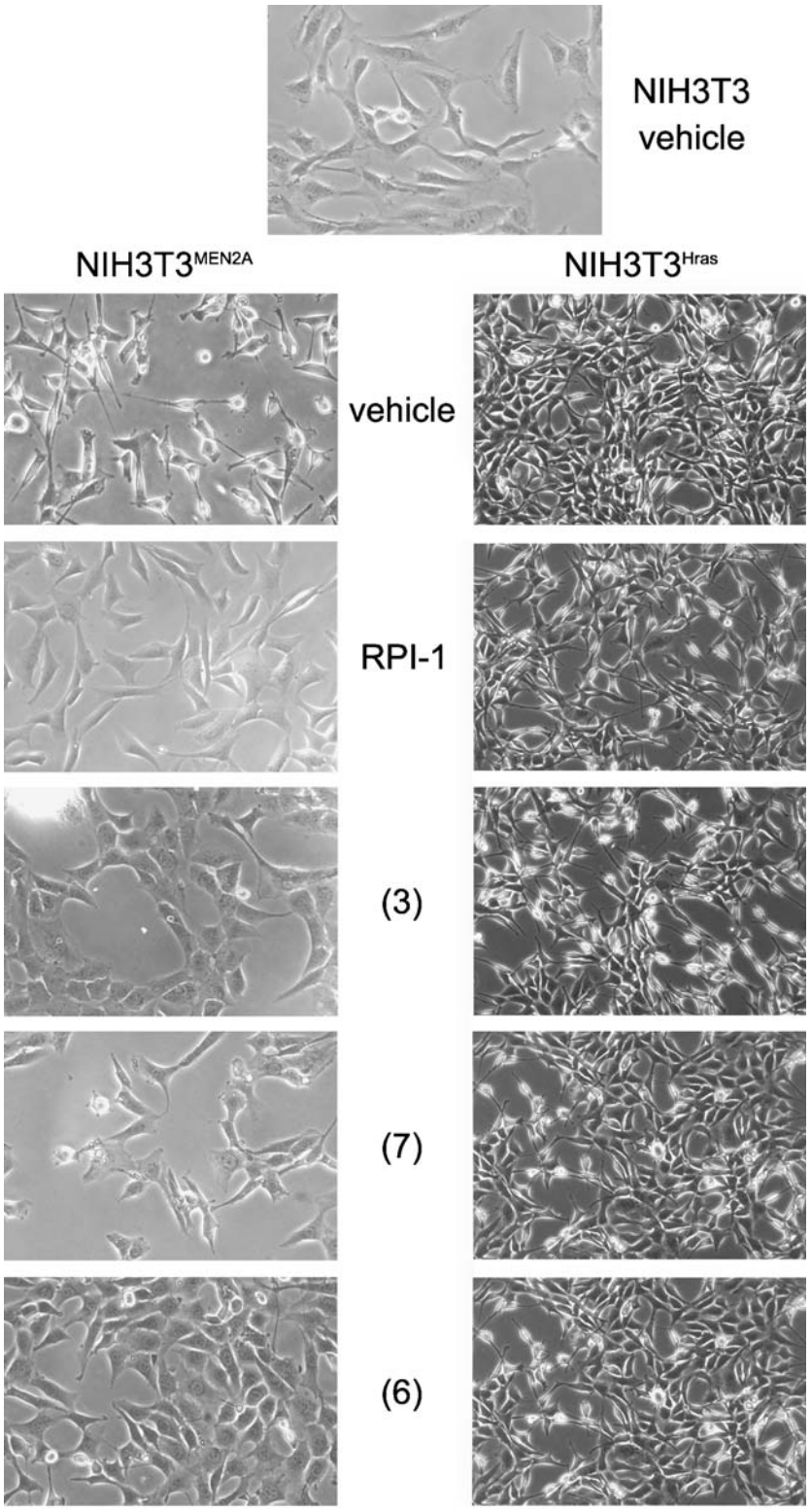


Figure 1. Effect of selected 3-arylureidobenzylidene-indolin-2-ones on the transformed morphologic cell phenotype of murine fibroblasts NIH3T3 transfected with RETC634R (NIH3T3^{MEN2A}) or H-RAS oncogene (NIH-3T3$^{H\text{-}RAS}$). Cells were treated with vehicle or 10 μM compound, then photographed under a phase-contrast microscope after 24 h (NIH3T3^{MEN2A}) or 72 h (NIH-3T3$^{H\text{-}RAS}$) of treatment. Parental NIH3T3 cells are shown for comparison. Original magnification 100×.

(a) **3**, characterized by an unsubstituted distal aryl group, retained activity features, including direct Ret kinase inhibitory activity, comparable to those of RPI-1.

(b) The presence of substituents in the distal aryl moiety reduced the ability to inhibit Ret function in cell culture, as indicated by the less persistent effects of **6** and **7** on transformed morphology. This is

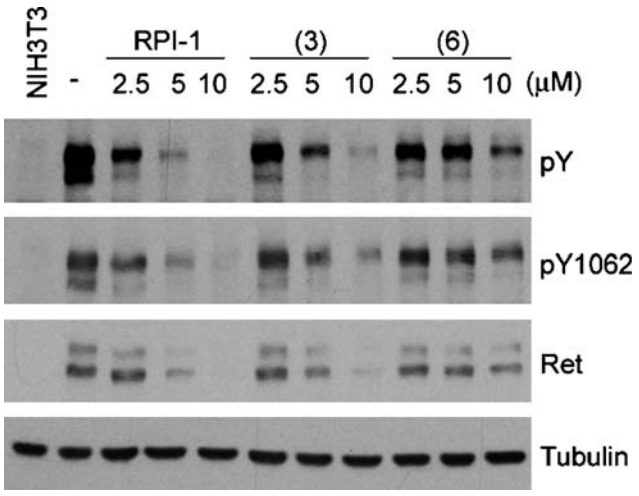


Figure 2. Effect of treatment with compounds **3**, **6** and RPI-1 reference compound on Ret autophosphorylation and expression. NIH3T3^{MEN2A} cells were exposed to vehicle or to the indicated concentrations of drugs for 24 h. Whole cell lysates were analyzed by Western blotting with the indicated antibodies. pY, generic pTyr; pY1062, specific pRet-Tyr1062.

consistent with the loss of activity of compounds with bulkier substituents (e.g., derivatives **8** and **9**). On the other hand, the presence of hydroxyl group in the distal aryl (compound **6**) conferred the highest inhibitory activity on Ret enzyme in cell-free system. In fact, the *O*-methyl derivative of compound **6** (**7**) was found ten times less potent.

(c) Methylation of the indolin-2-one nitrogen of **3** causes loss of Ret inhibitory activity and specificity against Ret-transformed cells.

(d) The replacement of the distal aryl moiety by a basic piperazine (**12**) did not allow persistent cellular effect, probably as a consequence of a reduced interaction with the target, as indicated by the Ret kinase assay.

(e) Hydrogenation of the exocyclic double bond (**14** vs. **3**), with the consequent loss of coplanarity of the rings, strongly depressed the activity.

(f) Finally, the presence of the two methoxy groups at positions 5 and 6 in the indolin-2-one nucleus seems to play an important role in the activity (compare **3** vs **1b**).

In conclusion, the present study has identified new inhibitors of Ret kinase in a series of 3-arylureidobenzylidene-indolin-2-ones. Structure–activity relationship evidences a favourable biochemical and biological activity activity of 5,6-dimethoxyindolinone derivatives with H, OH, or OMe in the *para* position of the distal aryl ring. Further studies are needed to optimize chemical–physical properties as well as the potency of these inhibitors.

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24. *General procedure for the synthesis of compounds* **1**–**14**. The appropriate aldehyde (1 equiv) was added to EtOH (3 ml/0.2 mmol) and the mixture was stirred until complete solution. The oxindole (1 equiv) and piperidine (0.1 equiv) were added, and the mixture was heated to 90 °C for 3–7 h, and cooled. The resulting precipitate was filtered, washed with cold ethanol and dried to give the pure compound. *General procedure for the synthesis of intermediate aldehydes*. 4-Formylphenylisocyanate (1 equiv) was suspended in toluene (2 M) and the appropriate aniline (1 equiv) was added. The mixture was refluxed for 2–5 h, then the solvent was removed by rotatory evaporation and the residue purified by flash chromatography on silica gel (CH_2Cl_2/MeOH). The compound (intermediate Schiff base) was dissolved in THF and 1 M HCl was added to the stirred solution. After 1 h at room temperature, THF was removed and the resulting precipitate was filtered, washed with water and dried to give the pure compound. *Spectral data for compounds* **1**–**14**. Compound **1a**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.60 (s, 1H, NH indol), 9.01 (s, 1H, –NH), 8.78 (s, 1H, –NH), 8.44 (d, J = 8.56 Hz, 2H, 2Ar), 7.60–7.75 (m, 2H, 2Ar), 7.50–7.60 (m, 2H, 2Ar), 7.45–7.50 (m, 2H, 2Ar), 7.25–7.40 (m, 2H, 1Ar + CH═), 7.15–7.25 (m, 1H, 1Ar), 6.90–7.10 (m, 2H, 2Ar), 6.75–6.80 (m, 1H, 1Ar). Compound **1b**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.55 (s, 1H, NH indol), 9.05 (s, 1H, –NH), 8.80 (s, 1H, –NH), 7.61–7.67 (m, 3H, 3Ar), 7.50–7.65 (m, 2H, 2Ar), 7.45–7.50 (m, 2H, 2Ar), 7.20–7.40(m, 4H, 3Ar + CH═), 6.85–7.10 (m, 3H, 3Ar). Compound **2**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.10 (s, 1H, NH indol), 9.15 (s, 1H, –NH), 8.80 (s, 1H, –NH), 8.46 (m, 2H, 2Ar), 7.78–7.95 (m, 2H, 2Ar), 7.40–7.65 (m, 4H, 4Ar), 7.10–7.35 (m, 3H, 2Ar + CH═), 6.95–7.10 (m, 1H, 1Ar), 6.75–6.82 (m, 1H, 1Ar). Compound **3**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.20 (s, 1H, NH indol), 8.42 (s, 1H, –NH), 8.21 (s, 1H, –NH), 7.60–7.75 (m, 4H, 4Ar), 7.50–7.54 (m, 2H, 2Ar), 7.41 (s, 1H, 1Ar), 7.35 (s, 1H, CH═), 7.22–7.26 (m, 2H, 2Ar), 6.98–7.01 (m, 1H, 1Ar), 6.60 (s, 1H, 1Ar), 3.82 (s, 3H, –OMe), 3.64 (s, 3H, OMe). Compound **4**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.01 (s, 1H, –NH), 8.76 (s, 1H, –NH), 7.69 (m, 2H, 2Ar), 7.61 (m, 2H, 2Ar), 7.45–7.52 (m, 3H, 3Ar), 7.25–7.35(m, 3H, 2Ar + CH═), 6.95–7.01 (m, 1H, 1Ar), 6.52 (s, 1H, 1Ar), 3.80 (s, 3H, –OMe), 3.65 (s, 3H, –OMe), 3.20 (s, 3H, -NMe). Compound **5**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.30 (s, 1H, NH indol), 9.00 (s, 1H, –NH), 8.81 (s, 1H, –NH), 7.67 (m, 2H, 2Ar), 7.61 (m, 2H, 2Ar), 7.48–7.52 (2H, m, 2Ar), 7.40 (s, 1H, 1Ar), 7.28 (s, 1H, CH═), 7.10–7.15 (m, 2H, 2Ar), 6.51 (s, 1H, 1Ar), 3.80 (s, 3H, –OMe), 3.62 (s, 3H, OMe). Compound **6**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.20 (s, 1H, NH indol), 9.10 (s,1H, –OH), 8.89 (s, 1H, –NH),8.44 (s, 1H, –NH), 7.67 (m, 2H, 2Ar), 7.61 (m, 2H, 2Ar), 7.37 (s, 1H, 1Ar), 7.28 (s, 1H, CH═), 7.23 (d, J = 8.56 Hz, 2H, 2Ar), 6.70 (d, J = 8.56 Hz, 2H, 2Ar), 6.51 (s, 1H, 1Ar), 3.78 (s, 3H, –OMe), 3.62 (s, 3H, OMe). Compound **7**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.30 (s, 1H, NH indol), 9.80 (s, 1H, –NH), 9.02 (s, 1H, –NH), 7.60–7.75(m, 4H, 4Ar), 7.42 (d, J = 8.56 Hz, 2H, 2Ar), 6.82 (d, J = 8.56 Hz, 2H, 2Ar), 6.51 (s, 1H, 1Ar), 3.80 (s, 3H, –OMe), 3.75 (s, 3H, –OMe)3.65 (s, 3H, –OMe). Compound **8**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.31 (s, 1H, NH indol), 8.86 (s, 1H, –NH), 8.40 (s, 1H, –NH), 7.67 (m, 2H, 2Ar), 7.58 (m, 2H, 2Ar), 7.37 (s, 1H, 1Ar), 7.27 (s, 1H, CH═), 7.25 (d, J = 8.56 Hz, 2H, 2Ar), 6.70 (d, J = 8.56 Hz, 2H, 2Ar), 6.51 (s, 1H, 1Ar), 3.79 (s, 3H, –OMe), 3.62 (s, 3H, OMe), 2.84 (s, 6H, NMe_2). Compound **9**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.30 (s, 1H, NH indol), 9.06 (s, 1H, –NH), 8.78 (s, 1H, –NH), 7.67 (m, 2H, 2Ar), 7.62 (m, 2H, 2Ar), 7.37 (s, 1H, 1Ar), 7.28 (s, 1H, CH═), 7.22 (d, J = 2.23 Hz, 1H, 1Ar), 6.84 (d, J = 8.56 Hz, 1H, 1Ar), 6.79 (dd, J = 2.23, 8.56 Hz, 1H, 1Ar), 6.51 (s, 1H, 1Ar), 5.98 (s, 2H, OCH_2O), 3.79 (s, 3H, –OMe), 3.62 (s, 3H, OMe). Compound **10**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.75 (s, 1H, NH indol), 9.07 (s, 1H, –NH), 8.83 (s, 1H, –NH), 8.44 (d, J = 8.56 Hz, 2H, 2Ar), 7.82 (s, 1H), 7.74 (d, J = 7.44 Hz, 1H, 1Ar), 7.58 (d, J = 8.56 Hz, 2H, 2Ar), 7.47 (d, J = 7.82 Hz, 2H, 2Ar), 7.23–7.37 (m, 3H, 2Ar + CH═), 6.89-7.11 (m, 3H, 3Ar). Compound **11**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.40 (s, 1H, NH indol), 9.25 (brs, 2H, 2-NH), 8.46 (m, 2H, 2Ar), 7.72 (m, 2H, 2Ar), 7.59 (m, 2H, 2Ar), 7.45 (m, 2H, 2Ar), 7.37 (s, 1H, 1Ar), 7.25 (s, 1H, CH═), 6.51 (s, 1H, 1Ar), 3.78 (s, 3H, –OMe), 3.62 (s, 3H, Ome). Compound **12**:. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.20 (s, 1H, NH indol), 7.75 (m, 2H, 2Ar), 7.55 (m, 2H, 2Ar), 7.48 (s, 1H, 1Ar), 7.10 (s, 1H, CH═), 6.50 (s, 1H, 1Ar), 3.78 (s, 3H, –OMe), 3.60 (s, 3H, OMe), 3.40–3.70 (m, 4H), 2.18–2.45 (m, 4H), 2.15 (s, 3H, -NMe). Compound **13**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.42 (s, 1H, NH indol), 10.35(s, 1H, –NH), 8.09 (d, J = 8.56 Hz, 2H, 2Ar), 7.84 (d, J = 8.56 Hz, 2H, 2Ar), 7.79 (d, J = 7.82 Hz, 2H, 2Ar), 7.47 (s, 1H, 1Ar), 7.35–7.39 (m, 2H, 2Ar), 7.12-7.18 (m, 2H, 1Ar + CH═), 6.53 (s, 1H, 1Ar), 3.80 (s, 3H, –OMe), 3.58 (s, 3H, OMe). Compound **14**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.20 (s, 1H, NH indol), 8.60 (s, 1H, –NH), 8.52 (s, 1H, –NH), 7.45 (m, 2H, 2Ar), 7.25–7.35 (m, 4H,4Ar), 7.00–7.15 (m, 2H, 2Ar), 6.80–6.95 (m, 1H, 1Ar), 6.55 (s, 1H, 1Ar), 6.45 (s, 1H, 1Ar), 3.70–3.90 (m, 1H), 3.75 (s, 3H, –OMe), 3.62 (s, 3H, OMe), 3.40–3.55 (m, 1H), 2.75–2.90 (m, 1H).
25. Sun, L.; Tran, N.; Tang, F.; App, H.; Hirth, P.; McMahon, G.; Tang, C. *J. Med. Chem.* **1998**, *41*, 2588.
26. *Cell culture and antibodies*. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% serum for NIH3T3^{MEN2A} or 10% calf serum (Colorado Serum Company, Denver, CO) for NIH3T3 and NIH3T3$^{H\text{-}RAS}$ cells and incubated at 37 °C in a 10% CO_2 atmosphere. Compounds, dissolved in DMSO, were diluted in cell culture medium (0.5% solvent final concentration). IC_{50}s were calculated from dose-response curves obtained by cell counting after 72 h of treatment. The effect of selected compounds on tyrosine phosphorylation and expression of the Ret oncoprotein was assessed by Western blot analysis of NIH3T3^{MEN2A} cells after 24 h of treatment as previously described.[20] The following antibodies were used: monoclonal anti-pTyr antibody clone 4G10 (Upstate Biotechnology, Lake Placid, NY); rabbit polyclonal anti-Ret H300 and anti-pRet (Tyr1062) (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-tubulin (Sigma Chemical Company, St. Louis, MO).
27. *Kinase assay*. A non-radioactive kinase assay was performed using recombinant Ret active protein (Upstate, Lake Placid, NY) and Myelin Basic Protein (MBP) (Sigma) as kinase substrate following the Upstate protocol

with some modifications. Briefly, Ret protein (30 ng), was incubated for 10 min at 30 °C in kinase buffer (11.6 mM MOPS, pH 7, 0.2 mM EDTA, 0.9 mM EGTA, 13.5 mM $MgCl_2$, 4.5 mM β-glycerophosphate, 0.18 mM Na_3VO_4 and 0.18 mM dithiothreitol) with solvent or inhibitor (DMSO 4% final concentration) in the presence of 2 μM MBP and 5 μM ATP. The reaction was stopped by 3-fold concentrated Laemmli buffer, then samples were subjected to SDS–PAGE and immunoblotted with anti-pTyr antibody. Phosphorylated MBP was revealed by chemioluminescence reaction ECL (GE Healthcare, Little Chalfont, UK) and detected by ChemiDoc XRS System, PC. Signal intensity was analyzed with the interfaced software Quantity One 4.6.3 (Bio-Rad, Hercules, CA).