Design, Synthesis and Biological Evaluation of New Potent and Highly Selective Ros1-Tyrosine Kinase Inhibitor

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Abstract – Ros1 protein is a receptor tyrosine kinase that has been reported mainly in meningiomas and astrocytomas, and until now, there is no selective inhibitor for this kinase. In this study, we illustrate for the synthesis of a highly potent and selective inhibitor for Ros1 kinase. The synthesized compound **1** was tested initially at a single dose concentration of 10 µM over 45 different kinases. At this concentration, a 94% inhibition of the enzymatic activity of Ros1 kinase was observed, while the inhibition in activity was below 30% in all of the other kinases. The pyrazole compound **1** was further tested in a 10-dose IC_{50} mode and showed an IC_{50} value of 199 nM for Ros1 kinase. Our compound **1** can be used as a promising lead for the development of new selective inhibitors for Ros1 kinase, and it may open the way for new selective therapeutics for astrocytomas.

Signal transduction is an essential biological process for normal cell growth and function. The transduction of many of these signals is mediated through growth factors which transmit their signals into the cell by a group of trans-membrane proteins with intrinsic tyrosine kinase activity, named receptor tyrosine kinases (RTKs).[1-4] Mutations at RTKs encoding genes are associated with the incidence of several types of cancers.[5-8] Ros1 is a human gene that encodes for a trans-membrane receptor tyrosine kinase.[9-11] It is located at the chromosome 6 region 6q16→6q22.[9] This region of chromosome 6 is involved in non-random chromosomal arrangements in specific neoplasias including acute lymphoblastic leukemias, malignant melanoma, and ovarian carcinoma.[9] A microdeletion at 6q21 of Ros1 results in the fusion of a golgi apparatus associated protein called FIG (Fused in Glioblastoma), to the kinase domain of the protoncogene Ros1 producing a chimeric protein with a constitutive receptor tyrosine kinase activity.[12,13]

This fused chimeric protein is a potent oncogene and its transforming potential lies in its ability to interact with golgi apparatus.[12]

The ectopic expression of Ros1 receptor protein has been reported mainly in meningiomas and astrocytomas (25% of low grade and 30% of malignant glioma tumors) suggesting a key role for Ros1 in these CNS malignancies.[12,14] A survey of 45 different human tumor cell lines, made by Birchmeier *et al.*,[11] has showed that Ros1 was expressed in glioblastoma-derived cell lines at high levels, while not expressed at all or expressed minimally in the remaining cell lines.[11]

Glioblastoma multiforme is the most advanced astrocytic neoplasm, and is one of the most aggressive human cancers with a median survival of less than one year. The complete resection of glioblastoma is impossible because of the diffuse infiltration of tumor cells into normal parenchyma. In addition, these tumors are highly resistant to radiation and chemotherapy. Despite decades of therapeutic research, effective chemotherapeutic treatment for high grade astrocytomas is not yet available, and patient care ultimately focuses on palliative management.[15-18]

Herein, we report the discovery of a potent and highly selective Ros1 RTK inhibitor **1** (Figure 1). The

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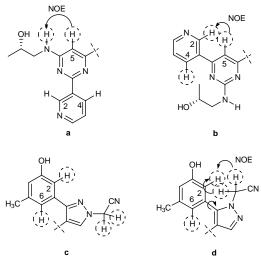 E-mail addresses: LSH6211@kist.re.kr

synthetic an d s creening protocols f or t he i nhibitor are illustrated in d etails. T he ki nase i nhibitory a ctivity of the s ynthesized c ompound was tested over 45 di fferent kinases, and it showed high selectivity for Ros1 kinase. According to o ur knowledge, th is is th e f irst s elective Ros1 tyrosine kinase inhibitor.

Figure 1. Structure of pyrazole compound **1**

For t he s ynthesis of t he t arget c ompound **1**, it w as important a t f irst to pr epare t he ke y e ster, methyl 3 - methoxy-5-methylbenzoate (**5**) a s illu strated in Scheme 1. The synthesis started with the preparation of the sodium s alt o f e thyl 2 -hydroxy-4-oxopent-2-enoate (**2**) according to lite rature procedure,[19] through t he condensation of diethyl oxalate w ith acetone in the presence of s odium e thoxide i n a bsolute ethanol. T he resulted s alt **2** was t hen c yclized i nto C laisen f uran derivative **3** by h eating in 5 0% acetic acid followed b y acidification with sulfuric acid.[20] The resulted C laisen compound un derwent r earrangement a nd a romatization into 3 -hydroxy-5-methylbenzoic aci d (**4**) w ithin le ss than one hour by he ating with magnesium oxi de i n boiling w ater, f ollowed b y a cidification w ith hydrochloric aci d to pr ecipitate t he pr oduct.[20] Methyl esterification a nd *O*-methylation o f th e r esulted phenolic aci d **4** were a chieved i n a s ingle s tep a nd i n high yield (94%) to give compound **5** through a little modification of t he l iterature pr ocedure,[21] where the acid **4** was r efluxed w ith e xcess pot assium c arbonate and i odomethane i n acet one i n t he p resence of a catalytic amount of DMAP.

In Scheme 2 , the benzoate ester **5** underwent a nucleophilic a ttack a t i ts carboxylic c arbon by t he activated methylene gr oup of 2,4 -dichloro-6-methyl-pyrimidine. The activation of this methyl group into an active methylene w as achieved by dropwise addition of lithium b is(trimethylsilyl)amide (LHMDS) in d ry T HF at room temperature. The resulted tautomeric α,β-unsaturated ke tone **6** was t hen s ubjected t o a nucleophilic substitution reaction with the amino group of (S)-(+)-1-aminopropan-2-ol by he ating a t 80 °C i n dry THF for 3 hours. Two regioisomers were produced from this reaction, but the major isomer was (*S*)-2-(6-(2-hydroxypropyl-amino)-2-chloropyrimidin-4-yl)-1-(3-methoxy-5-methylphenyl)ethanone (**7**), wh ich wa s separated in a pure form using column chromatography. The st ructure of t his i somer w as c onfirmed by the 2D-

NOESY NM R s pectrum of t he s ubsequent c ompound **10**. The conversion of the resulted tautomeric product **7** to t he r equired p yrazole d erivative **8** was ach ieved through t wo su ccessive st eps. I n t he f irst st ep, compound **7** was h eated w ith ex cess *N,N*-dimethylformamide dimethylacetal for 20 hours, and the resulted pr oduct w as t aken t o t he ne xt s tep w ithout further p urification, w here it w as c yclized w ith hydrazine m onohydrate i n a bsolute e thanol i nto t he pyrazole d erivative **8**. T he r eaction of t he r esulted pyrazole **8** with io doacetonitrile in the p resence o f excess pot assium c arbonate pr oduced t wo di fferent regioisomers. T he i ntended i somer **9** was th e m ajor product of t he r eaction w ith R f value of 0.54 (EtOAc) while the other isomer was produced as a minor product with hi gher R f value of 0.66 (EtOAc). T he r equired isomer **9** was s eparated i n a p ure f orm b y co lumn chromatography and its structure was confirmed by 2D-NOESY NM R s pectrum of t he s ubsequent c ompound **10**. C ompound **10** was prepared i n a m oderate y ield (67%) by S uzuki c oupling of c ompound **9** with 3 - pyridineboronic a cid, i n the pr esence of di chloro bis(triphenylphosphine)Pd(II) and s odium c arbonate, in a mixed solvent of acetonitrile and water in a (1:1) ratio. The 2D -NOESY NM R spectrum of t his c ompound confirmed the former nucleophilic substitution with (*S*)- (+)-1-aminopropan-2-ol in compound **7** at the 6-position of t he py rimidine r ing (not t he 2 -position). T his w as proved by the absence of a cross peak between H_5 of the pyrimidine r ing a nd H_2 or H 4 of t he py ridyl gr oup (Figure 2a).

a: NOE effect between aminopropan-2-ol NH and Pyrimidine H_5 in compound **10**; **b**: Proposed NOE effect between pyrimidine H_5 and H_2 or H_4 of pyridine in compound **10** regioisomer; **c**: The distance between acetonitrile -CH_2- and the aromatic protons doesn't allow for NOE interaction in compound **10**; **d**: Proposed NOE effect between acetonitrile -CH_2- and the aromatic protons in the *2H*-pyrazole isomer of compound **10**.

Figure 2. NOE interactions in compound 10 and its proposed isomers.

Scheme 1. Reaction conditions and reagents: (i) NaOEt, abs. EtOH, rt, 4 h, 87%; (ii) acetic acid : H_2O (1:1), rt, 2 h, 50%; (iii) MgO, H_2O, reflux, 45 min, 42%; (iv) K_2CO_3, CH_3I, DMAP, acetone, 65 ^{o}C, 12 h, 94%.

Scheme 2. Reaction conditions and reagents: (i) LHMDS, THF, N_2, rt, 24 h, 50%; (ii) THF, 80 ^{o}C, 3 h, 35%; (iii) DMF-DMA, 90 ^{o}C, 20 h; (iv) hydrazine hydrate, abs. EtOH, rt, 12 h, 44%; (v) K_2CO_3, iodoacetonitrile, acetone, reflux, 2 h, 43%; (vi) 3-pyridineboronic acid, $Pd(PPh_3)_2Cl_2$, Na_2CO_3, N_2, CH_3CN/H_2O (1:1), 78 ^{o}C, 3 h, 67%; (vii) $BF_3 \cdot S(CH_3)_2$, dichloromethane, N_2, rt, 12 h, 45%.

The presence of such cross peak would be expected if the substitution with (*S*)-(+)-1-aminopropan-2-ol occurred at the C_2 of the pyrimidine, since in this case, the subsequent substitution with pyridin-3-yl moiety would happen at the 6 position of pyrimidine, allowing through space interaction between H_5 of the pyrimidine and H_2 or H_4 of the pyridine (Figure 2b). Furthermore, the presence of a cross peak between pyrimidine H_5 and the NH of the aminopropan-2-ol provides another evidence for the presence of the aminopropan-2-ol group at the 6 position of the pyrimidine ring (Figure 2a). It was important also to prove that the former isomer **9** is the intended 1H-pyrazole not the 2H-pyrazole. This was proved too by the 2D-NOESY NMR spectrum of compound **10**, by the absence of any cross peak that might indicate a through space interaction between the –CH_2- protons of the acetonitrile group and any of the aromatic protons of the 3-methoxy-5-methylphenyl group (Figure 2c). The presence of such cross-peaks would be expected if this isomer is the 2H-pyrazole, since the acetonitrile group would be close enough to the aromatic protons of 3-methoxy-5-methylphenyl group to exhibit NOE effect as shown in Figure 2d. The final hydroxyl product **1** was obtained by demethylation of the methoxy group of compound **10** using 10 equivalents of boron trifluoride-dimethylsulfide complex in dichloromethane.

The screening results of the target compound **1** over 45 different kinases have revealed that the inhibitory activity of the compound was not exhibited over almost all of the tested kinases, while high potency and activity was selectively shown at Ros1 kinase only (Table 1).

Table 1. Percantages of enzymatic activities and inhibitions exerted by compound **1**[25] on 45 kinases[26]
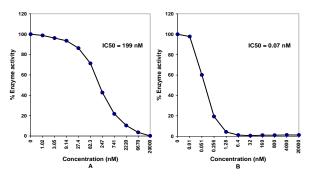
Kinase Enzyme	% Activity[a,b,c]	% Inhibition[d]
ABL1	79.56	20.44
AKT1 (dPH, S473D)	102.97	-2.97
Aurora A	78.25	21.75
BRAF	91.03	8.97
CDK1/cyclinB	96.36	3.64
CHK1	97.64	2.36
CK1epsilon	90.68	9.32
c-Kit	97.15	2.85
c-MET	94.18	5.82
c-Src	83.82	16.18
DAPK1	101.17	-1.17
DNA-PK	93.44	6.56
EGFR	102.23	-2.23
EPHA1	76.73	23.27
FAK/PTK2	96.57	3.43
FGFR1	108.05	-8.05
FGR	96.66	3.34
FLT1	101.67	-1.67
FYN	107.86	-7.86
HIPK1	103.43	-3.43
IKKa/CHUK	98.63	1.37
IR	92.99	7.01
JAK1	90.85	9.15
JNK1a1	96.17	3.83
KDR/VEGFR2	105.84	-5.84
LCK	89.72	10.28
LYN	101.45	-1.45
MEK1	106.97	-6.97
MST4	101.54	-1.54
MUSK	100.42	-0.42
P38a/MAPK14	94.73	5.27
p70S6K	94.21	5.79
PAK4	98.63	1.37
PIM1	94.09	5.91
PKCa	90.77	9.23
PLK1	94.15	5.85
RAF1	79.61	20.39
RET	93.35	6.65
ROCK1	88.28	11.72
RON/MST1R	71.08	28.82
ROS/ROS1	**6.08**	**93.92**
SYK	91.77	8.23
TIE2/TEK	91.83	8.17
TRKA/NTRK1	100.14	-0.14
YES	102.59	-2.59

[a] % activity in each enzyme is the mean of two different readings.
[b] Test compound was used in a single dose concentration of 10 μM.
[c] 100% activity refers to enzyme activity in negative control (DMSO).
[d] % Inhibition was calculated by subtracting % activity from 100.

The compound was tested initially at a single dose concentration of 10 μM. At this concentration, a 93.92%

inhibition of the enzymatic activity of Ros1 kinase was observed, while the inhibition in activity was below 30% in all of the other kinases, and in the range of 20-30% in 5 kinases only (ABL1, Aurora A, EPHA1, RAF1 & RON/MST1R).

Compound **1** was further tested over Ros1 kinase in order to determine its IC_{50}, where a 10-dose IC_{50} mode with 3 folds serial dilutions starting at 20 μM concentration was applied against Staurosporine[22-24] as a reference standard (Figure 3). The compound has showed an IC_{50} value of 199 nM, while the IC_{50} value for the non-selective kinase inhibitor Staurosporine was 0.07 nM.

(A) Dose-activity curve for compound **1** on Ros1 kinase
(B) Dose-activity curve for Staurosporine on Ros1 kinase
Figure 3. Dose-activity curves

The high selectivity of compound **1** to Ros1 kinase and the diminished activity over the other kinases could be attributed to the increased bulkiness exerted by the sbstituents at pyrimidine nucleus. This increase in bulkiness seems to hinder the fitting of the compound to the binding sites of most of these kinases and to exclude it from their binding pockets. However, the selective inhibition of Ros1 kinase might be owed to a unique difference in the geometry of the binding pocket of this enzyme that enables the fitting and interaction of compound **1**. An indirect inhibitory effect of compound **1** at Ros1 kinase, probably through binding at an allosteric binding site, is another possible assumption for the reason behind this selective inhibition. The absolute reason is still unclear and we believe that it worth further exploration for the mechanism of its unique inhibitory effect.

In conclusion, a highly potent and selective inhibitor for Ros1 kinase has been synthesized and can be used as a promising lead for new selective inhibitors for Ros1 kinase. It worth also to mention that until now, no selective inhibitor is available for Ros1 kinase, and the development of new selective inhibitors for such kinase might open the way for new selective therapeutics for astrocytomas. Screening of compound **1** against glioblastoma derived tumors is currently undergoing.

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25. *Preparation* of c ompound **1**: To a s olution o f compound **10** (75 mg, 0.16 mmol) in dichloromethane (3 mL) was a dded bor ontrifluoride-methyl s ulfide c omplex (172 µL, 1.65 mmol) in a dropwise manner at room temperature a nd u nder N $_2$ atmosphere. T he r esulting suspension was s tirred for 12 h ours, a nd t hen the mixture was co ncentrated u nder v acuum. T he r esidue was partitioned be tween e thylacetate (100 m L) a nd br ine (50 mL). T he o rganic l ayer was s eparated an d d ried over anhydrous M gSO$_4$, t hen e vaporated und er va cuum. T he residue w as p urified b y co lumn c hromatography (silica gel, methanol-dichloromethane 1:30, v/v) to yield the pure hydroxyl product **1** as a white powder (32.5 mg, 45%); mp 236-237 $^{\circ}$C; ^{1}H-NMR (CD$_3$OD) δ 1.09 (d, J = 5.7 Hz, 3H), 2.21 (s, 3H), 3.25 (s, 1H), 3.38 (bs, 1H), 3.87 (bs, 1H), 5.32 (s, 2H), 6.65 (s, 1H), 6.69 (s, 1H), 6.76 (s, 1H), 6.86 (s, 1H), 7.38 – 7.41 (m, 1H), 8.14 (d, J = 8.1 Hz, 1H), 8.28 (s, 1H), 8.49 (d, J = 3.5 Hz, 1 H), 8.81 (s, 1 H); IR (KBr) 3425, 2926, 2360, 1586, 1454, 1349, 1162 cm^{-1}.
26. Kinase a ssays were p erformed at R eaction B iology Corporation u sing t he "HotSpot" a ssay pl atform. Kinase Assay Protocol. Reaction Buffer: base Reaction buffer; 20 mM Hepes (pH 7.5), 10 mM MgCl$_2$, 1 mM EGTA, 0.02% Brij35, 0.02 m g/ml B SA, 0.1 mM N a$_3$VO$_4$, 2 mM D TT, 1% DMSO. Required cofactors were added individually (if needed) to each k inase reaction. R eaction Procedure: To a freshly pr epared bu ffer solution was a dded a ny r equired cofactor f or t he en zymatic r eaction, f ollowed b y t he addition of the selected kinase at a concentration of 20 µM. The contents were mixed gently, then the compound under test (compound **1**) di ssolved i n D MSO was a dded t o t he reaction mixture i n t he ap propriate co ncentration. 3 39-ATP (specific a ctivity 5 00 µCi/µl) was added to the mixture i n o rder t o i nitiate t he r eaction, an d t he mixture was i ncubated a t r oom t emperature f or 2 h ours. Initial screening o ver 4 5 ki nases: Compound **1** was t ested b y single dose d uplicate made at a concentration o f 10 µ M. Staurosporine was used as a control compound in a 5-dose IC$_{50}$ mode with 1 0 fold s erial di lutions s tarting at 2 0 µ M. Reaction was car ried o ut a t 1 0 µM A TP co ncentration. Testing a gainst Ros1 kinase: Compound **1** was tested in a 10-dose IC$_{50}$ mode with 3 fold s erial d ilutions starting a t 20 µM. Staurosporine was used as a control compound in a 10-dose IC$_{50}$ mode with 5 fold s erial d ilutions starting a t 20 µM . R eaction was car ried o ut at 1 0 µM ATP concentration.