Journal Pre-proofs

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Identification of 4-Anilino-6-aminoquinazoline Derivatives as Potential MERS-CoV Inhibitors

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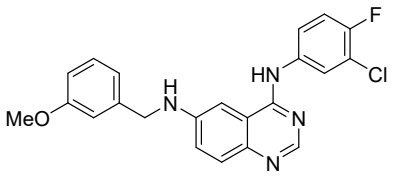
Abstract : New therapies for treating coronaviruses are urgently needed. A series of 4-anilino-6-aminoquinazoline derivatives were synthesized and evaluated to show high anti-MERS-CoV activities. *N*^{4}-(3-Chloro-4-fluorophenyl)-*N*^{6}-(3-methoxybenzyl)quinazoline-4,6-diamine (**1**) has been identified in a random screen as a hit compound for inhibiting MERS-CoV infection. Throughout optimization process, compound **20** was found to exhibit high inhibitory effect (IC_{50} = 0.157 μM, SI = 25) with no cytotoxicity and moderate *in vivo* PK properties.

Coronaviruses (CoVs) are a group of positive-sense, single-stranded RNA viruses that cause severe respiratory diseases in a broad range of animal species, including humans.[1-3] In 2003, one of the novel coronaviruses, severe acute respiratory syndrome CoV (SARS-CoV), caused a total of 8,422 cases of SARS with 916 deaths.[4] The other novel coronavirus, Middle East respiratory syndrome CoV (MERS-CoV), has emerged in April 2012 and posed a serious threat to public health. As of 4 April 2020, a total of 2,494 human MERS-CoV infections with 858 deaths had been reported from 27 countries.[5] Although MERS-CoV can cause primary infections from direct contact with animal reservoirs like camels,[6] person-to-person transmission of this virus has mainly occurred in health-care facilities and family clusters.[7-9] Recently outbreak of COVID-19, which is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in China and has spread to several other countries.

Drug repositioning for an FDA-approved compound library found that numerous compounds inhibited MERS-CoV infection. However, there are still no approved drugs for coronaviruses.[10] Through high content screening (HCS) platform of Institut Pasteur Korea (IPK) using the Korea Chemical Bank (KCB),[11] we found several novel compounds that can inhibit MERS-CoV infection.[12] We found that *N*^{4}-(3-chloro-4-fluorophenyl)-*N*^{6}-(3-methoxybenzyl)quinazoline-4,6-diamine **1** was effective for inhibiting MERS-CoV infection. Quinazoline derivatives have previously been reported as potent inhibitors of the protein kinase of epidermal growth factor receptor (EGFR).[13] Most of those compounds were approved as anticancer drugs, including Gefitinib,[14] Lapatinib,[15] Erlotinib,[16] and Afatinib[17]. We thought that quinazoline compounds can exhibit good bioavailability and be easily extended to treatment of MERS-CoV infection. Here we report on the synthesis and biological effects of 4-anilino-6-aminoquinazoline derivatives.

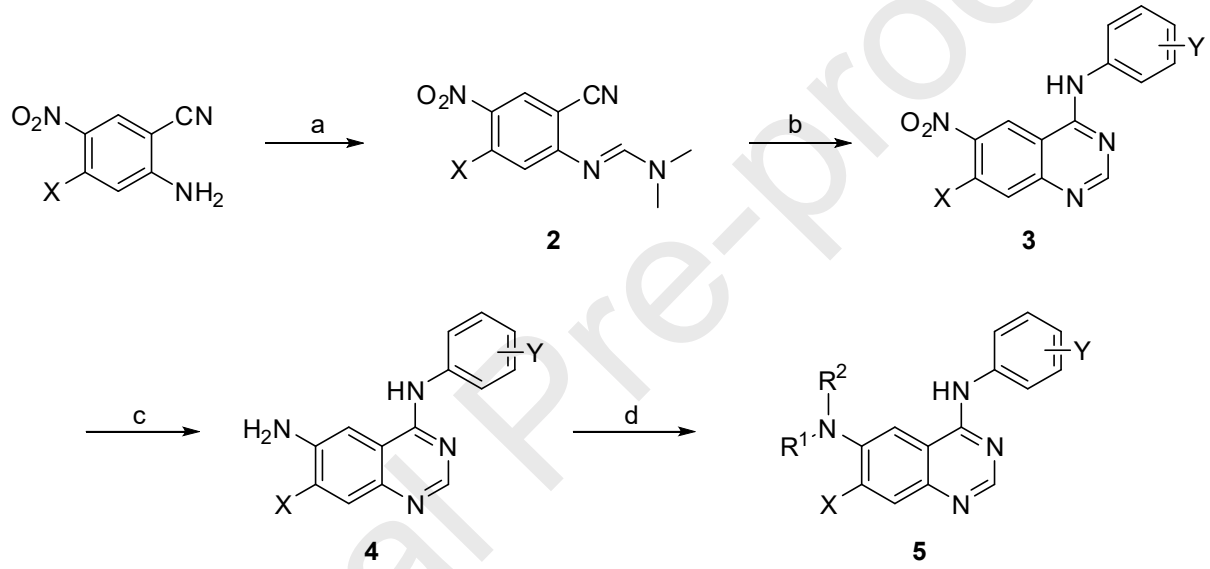
The general synthetic route for 4-aminoquinazoline derivatives is described in Scheme 1. 2-Amino-5-nitrobenzonitrile was reacted with dimethylformamide-dimethyl acetal (DMF-DMA) in toluene to give (*E*)-*N'*-(2-cyano-4-nitrophenyl)-*N*,*N'*-dimethylformimidamide **2**. *N*,*N*-dimethylformamidine **2** was treated with acetic acid and anilines at 120 ^{o}C to produce 6-nitroquinazoline **3**.[18] Reduction of the nitro group of **3** was carried out using iron powder and NH_4Cl in isopropyl alcohol and water at 100 ^{o}C to afford 6-aminoquinazoline **4**. Aromatic amine **4** was reductive alkylated with aldehydes using $NaBH(OAc)_3$ and trifluoroacetic acid in isopropyl acetate at 100 ^{o}C to give *N*-substituted quinazolines **5**.



1

IC_{50} : 5.6 μM
CC_{50} : > 25 μM
SI : 4.5

Figure 1. Hit against MERS-CoV from a KCB library screen



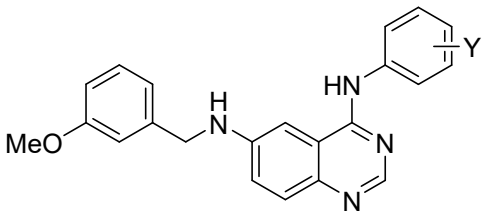
2 **3** **4** **5**

Scheme 1. Synthesis of 6-substituted 4-anilinoquinazoline derivatives. Reagents and conditions: (a) DMF-DMA, toluene, 110 °C, 5 h; (b) $ArNH_2$, AcOH, 120 °C, 7 h; (c) Fe, NH_4Cl, i-PrOH/H_2O (10/1), 110 °C/4 h; (d) Aldehyde, $NaBH(OAc)_3$, trifluoroacetic acid, i-PrOAc, 90 °C, 3 h; Acyl chloride, Et_3N, dichloromethane, rt; or carboxylic acid, EDCI, i-Pr_2NEt, DMF, rt.

The antiviral activities of the synthesized compounds in Vero cells were determined by immunofluorescence assay (IFA). Vero cells were infected with a korean clinical MERS-CoV isolates and the inhibitory concentration (IC_{50}) and cytotoxic concentration (CC_{50}) values of the compounds were calculated by nonlinear regression analysis.[11] Molecules with higher selective index (SI) were considered as active molecules.

Our preliminary structure activity relationship (SAR) studies surrounding **1** were carried out by introducing halide groups on phenyl ring of 4-anilino group (Table 1). 4-Bromo substituent (**6**) maintained the potency. 4-Fluoro (**7**), 3-chloro (**8**), 4-cyano (**9**), and 3-acetyl (**10**) reduced potency compared to **1**. Interestingly, 4-trifluoromethyl (**11**) can slightly improve the potency. In the next phase optimization, we observed the effects of the substituents of 6 position of quinazoline ring, having fixed with 3-chloro-4-fluoro and 4-trifluoromethyl substituents on phenyl ring at 4 position (Table 2).

Table 1. Antiviral effect and toxicity of 4-anilino groups of hit compound **1**



Compound	Y	IC_{50}(μM)[a]	CC_{50}(μM)[b]	SI[c]
1	3-Cl,4-F	5.6	>25	4.5
6	4-Br	7.4	>25	3.4
7	4-F	22.7	>25	1.1
8	3-Cl	>25	23.0	1.0
9	4-CN	>25	>25	1.0
10	3-$COCH_3$	>25	>25	1.0
11	4-CF_3	1.8	>25	1.0

[a,b] IC_{50} and CC_{50} were derived from the results of at least two dependent experiment in Vero cells infected with MERS-CoV.

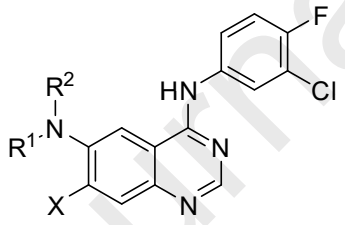
[c] SI(selective index) = CC_{50}/IC_{50} for inhibiting MERS-CoV infection.

First, substituent effects at 6 position of **1** having 3-chloro-4-fluoro aniline at 4 position were evaluated. Changing 3-methoxybenzyl amine to 2-methoxybenzyl amine group **12** showed similar activity (IC_{50} = 3.8 μM). Introducing 2-hydroxybenzyl amine at 6 position of quinazoline ring **13**–was tolerated (IC_{50} = 3.6 μM). 3,4-Difluoro (**14**) and 4-fluoro (**15**) compounds gave similar activities (IC_{50} = 4.6 μM). We next explored the effects of electron-withdrawing groups. Compounds with nitro groups (**16** to **18**) showed similar inhibitory effects (IC_{50} = 3.3, 6.1 and 2.7 μM, respectively). Compound with 2-cyanobenzyl amine at 6 position

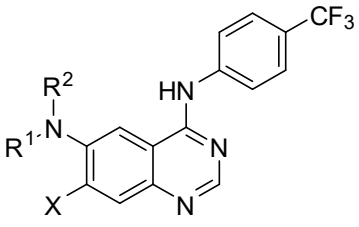
(**19**) showed no inhibitory effect, whereas 4-cyanoebnzyl amine (**21**) functionality was tolerated (IC_{50} = 4.8 μM). Gratifyingly, 3-cyanobenzyl amine analogue **20** resulted in significant higher activity (IC_{50} = 0.157 μM). We tested other electron-withdrawing groups at meta position of benzyl amine substituent. 3-Amidobenzyl amine analogue **22** was detrimental for activity but trifluoromethyl substituent at 3 position **23** retained the inhibitory effect (IC_{50} = 2.3 μM). Then benzyl amine substituents at 6 position were changed to aliphatic amines and amides (**24**–**28**). Aliphatic amine substituents, such as di-n-butyl (**24**) and cyclohexyl (**25**), showed little inhibitory effects and amide groups (**26**–**28**) were detrimental. We introduced several substituents at 7 position of **20**. Methoxy at 7 position gave a little decreased activity (**29**, IC_{50} = 5.8 μM) but hydroxy (**30**) showed no inhibitory effects.

The antiviral effects of derivatives at 6 position of **11** with 4-trifluoromethyl aniline group at 4 position were also examined. Most of substituents were well tolerated. 3-Cyanobenzyl (**32**), 4-cyanobenzyl (**33**), 3-nitrobenzyl (**34**) and 4-nitrobenzyl (**35**) showed similar activities (IC_{50} = 3.3 to 4.1 μM) to **11**. However, 2-cyanobenzyl (**31**) substituent exhibited decreased potency.

Table 2. Structure-Activity Relation of Derivatives of **1** and **11**



12-31



32-36

Compound	X	R_1	R_2	IC_{50}(μM)[a]	CC_{50}(μM)[b]	SI[c]
1	H	3-CH_3O-$PhCH_2$	H	5.6	>25	4.5
12	H	2-CH_3O-$PhCH_2$	H	3.8	>25	7
13	H	2-OH-$PhCH_2$	H	3.6	>25	7
14	H	3,4-F_2-$PhCH_2$	H	4.6	23.0	5
15	H	4-F-$PhCH_2$	H	4.6	14.4	3
16	H	2-NO_2-$PhCH_2$	H	3.3	>25	8
17	H	3-NO_2-$PhCH_2$	H	6.1	>25	4

18	H	4-NO_2-$PhCH_2$	H	2.7	>25	8
19	H	2-CN-$PhCH_2$	H	>25	>25	1
20	H	3-CN-$PhCH_2$	H	0.157±0.002	3.59±1.1	25
21	H	4-CN-$PhCH_2$	H	4.8	>25	5
22	H	3-H_2NCO-$PhCH_2$	H	>25	>25	1
23	H	3-CF_3-$PhCH_2$	H	2.3	9.2	4
24	H	n-butyl	n-butyl	>25	>25	1
25	H	cyclohexyl	H	15	>25	1
26	H	CH_3CO	H	>25	>25	1
27	H	3-CN-PhCO	H	>25	>25	1
28	H	3-CN-trans-cynnamoyl	H	>25	>25	1
29	OMe	3-CN-$PhCH_2$	H	5.8	13.2	2
30	OH	3-CN-$PhCH_2$	H	>25	>25	1
11	H	3-CH_3O-$PhCH_2$	H	1.8	>25	1
31	H	2-CN-$PhCH_2$	H	>25	>25	1
32	H	3-CN-$PhCH_2$	H	3.6	>25	7
33	H	4-CN-$PhCH_2$	H	4.1	>25	6
34	H	3-NO_2-$PhCH_2$	H	3.7	>25	7
35	H	4-NO_2-$PhCH_2$	H	3.3	>25	8

[a,b] IC_{50} and CC_{50} were derived from the results of at least two dependent experiment in Vero cells infected with MERS-CoV.

[c] SI(selective index) = CC_{50}/IC_{50} for inhibiting MERS-CoV infection.

Considering activity and cytotoxicity, **20** was thought to be the best compounds for anti-MERS drug and evaluated for its hERG, metabolic stability, cytotoxicity (Table 3). Our optimized lead compound **20** was found to show no hERG binding and have good microsomal stability in both mouse and human. Compound **20** showed no cytotoxicity toward Vero (not infected with MERS-CoV) as well as toward HFL-1, L929 NIH 3T3 and CHO-K1 cell lines, which shows that potential interaction between compound **20** and viruses might affect the cell viability. The pharmacokinetic parameters of **20** were evaluated in rats by intravenous (i.v.)

and oral (p.o.) routes at 2 and 5 mg/kg, respectively (Table 4). **20** showed reasonable oral bioavailability (21 %)

Table 3. Result of hERG, Microsomal stability, cytotoxicity of **20**

Compound	hERG (μM)	MS[a]		Cytotoxicity (μM)[b]				
		m[a]	h[a]	Vero[c]	HFL-1	L929	NIH 3T3	CHO-K1
20	>50	60.7	56.4	>100	>100	>100	>100	>100

[a] % original compound remained after 30 min incubation

[b] Cell information. Vero: African green monkey kidney cell line, HFL-1: human embryonic lung cell line, L929: NCTC clone 929, mouse fibroblast cell line, NIH 3T3 : mouse embryonic fibroblast cell line, CHO-K1 : Chinese hamster ovary cell line.

[c] Vero cell was not infected with MERS-CoV.

Table 4. *In vivo* pharmacokinetic profiles in rat of **20**

Parameters*	**I.V., 2 mg/kg**	**P.O., 5 mg/kg**
Tmax (h)	NA**	2.0
Cmax (μg/h)	NA	0.2
$T_{1/2}$ (h)	5.9	5.5
AUC (μg·h/mL)	1.11	0.57
CL (L/h/kg)	1.73	NA
V_{ss} (L/Kg)	6.3	NA
F_t (%)	NA	21

*All results are the mean of experiments using three rats.

**NA : not applicable

In conclusion, we have synthesized a series of 4-anilino-6-aminoquinazoline derivatives and most of the compounds showed anti-MERS-CoV activity in Vero cell. The best compound among the derivatives was **20**, which had the form of quinazoline with 3-Chloro-4-

fluoroaniline at 4 position and 3-cyanobenzyl amine at 6 position. Compound **20** showed high anti-MERS-CoV activity (IC_{50} = 0.157 μM, SI = 25) with no cytotoxicity and moderate *in vivo* PK property. Further studies on additional SAR and pharmacological investigation of these compounds are currently underway.

Acknowledgements

The chemical library used in this study was kindly provided by Korea Chemical Bank (http://www.chembank.org/) of Korea Research Institute of Chemical Technology. This work was supported by a grant of National Research Council of Science & Technology (NST) by the Korean government (MSIP) (No. CRC-16-01-KRICT).

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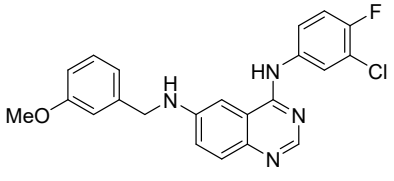
Declaration of interests

■ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Chul Min Park

19.



1

IC_{50} : 5.6 μM
CC_{50} : > 25 μM
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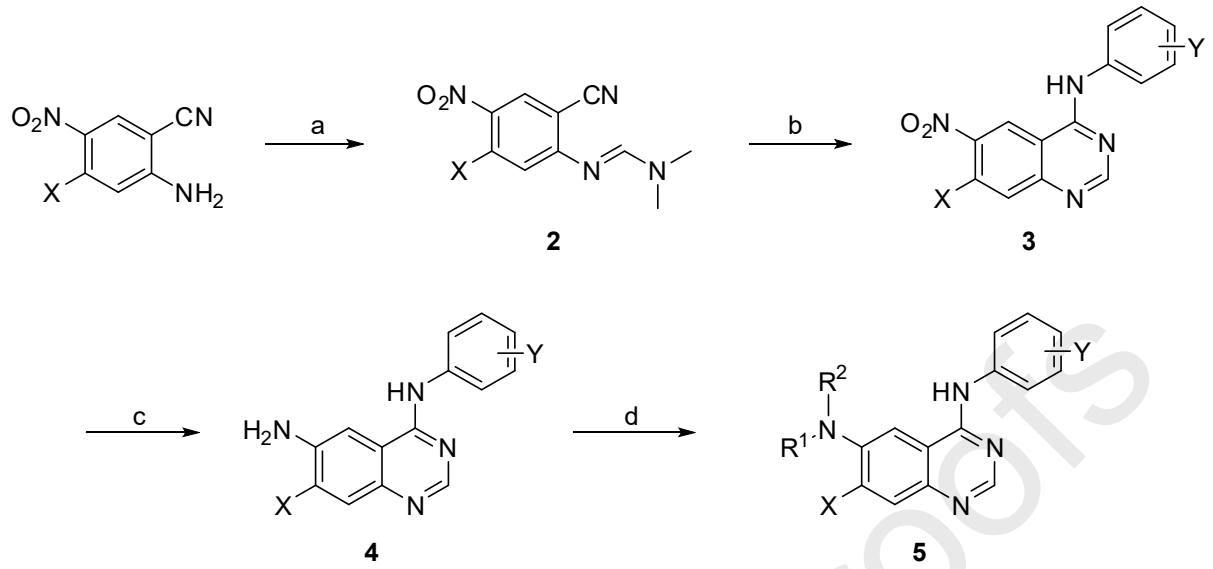
Figure 1. Hit against MERS-CoV from a KCB library screen



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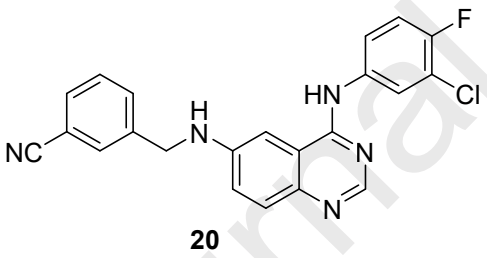
20.



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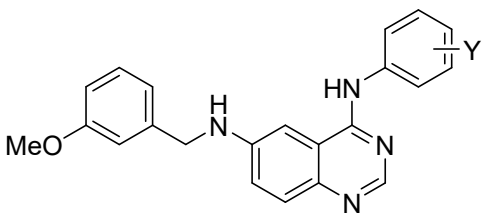

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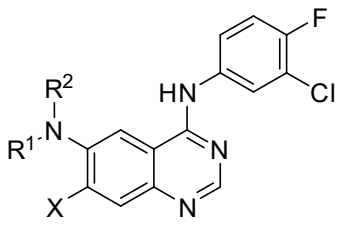

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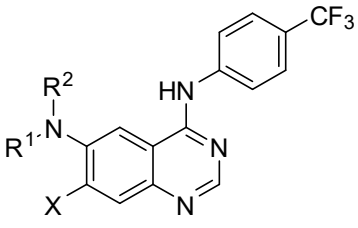

32-36

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22	H	3-H_2NCO-PhCH_2	H	>25	>25	1
23	H	3-CF_3-PhCH_2	H	2.3	9.2	4
24	H	n-butyl	n-butyl	>25	>25	1
25	H	cyclohexyl	H	15	>25	1
26	H	CH_3CO	H	>25	>25	1
27	H	3-CN-PhCO	H	>25	>25	1
28	H	3-CN-trans-cynnamoyl	H	>25	>25	1
29	OMe	3-CN-PhCH_2	H	5.8	13.2	2
30	OH	3-CN-PhCH_2	H	>25	>25	1
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31	H	2-CN-PhCH_2	H	>25	>25	1
32	H	3-CN-PhCH_2	H	3.6	>25	7
33	H	4-CN-PhCH_2	H	4.1	>25	6
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[a] % original compound remained after 30 min incubation

[b] Cell information. Vero: African green monkey kidney cell line, HFL-1: human embryonic lung cell line, L929: NCTC clone 929, mouse fibroblast cell line, NIH 3T3 : mouse embryonic fibroblast cell line, CHO-K1 : Chinese hamster ovary cell line.

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Table 4. *In vivo* pharmacokinetic profiles in rat of **20**

Parameters*	**I.V., 2 mg/kg**	**P.O., 5 mg/kg**
Tmax (h)	NA**	2.0
Cmax (μg/h)	NA	0.2
$T_{1/2}$ (h)	5.9	5.5

AUC (μg·h/mL)	1.11	0.57
CL (L/h/kg)	1.73	NA
V_{ss} (L/Kg)	6.3	NA
F_t (%)	NA	21

*All results are the mean of experiments using three rats.

**NA : not applicable

22.