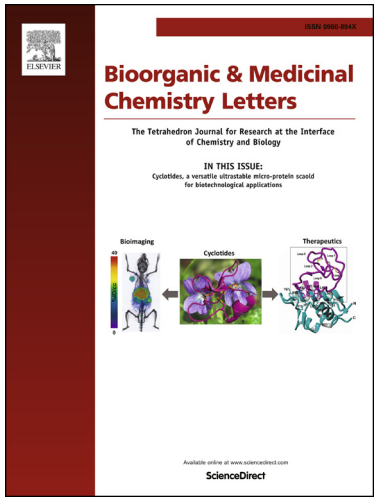
Accepted Manuscript



Discovery of Selective 2,4-Diaminoquinazoline Toll-like Receptor 7 (TLR 7) Agonists

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Discovery of Selective 2,4-Diaminoquinazoline Toll-like Receptor 7 (TLR 7) Agonists

Serge Pieters,[a]* David McGowan,[a]Florence Herschke,[a]Frederik Pauwels,[a]Bart Stoops,[a]Stefaan Last,[a]Werner Embrechts,[a]Annick Scholliers,[a]Wendy Mostmans,[a]Kris Van Dijck,[a]Bertrand Van Schoubroeck,[a]Tine Thoné,[a]Dorien De Pooter,[a]Gregory Fanning,[a]Mari Luz Rosauro,[c] Mourad Daoubi Khamlichi,[c] Yannis Houpis,[a]Eric Arnoult,[b]Tim H. M. Jonckers,[a]Pierre Raboisson[a]

[a]Janssen Infectious Diseases Diagnostics BVBA, Turnhoutseweg 30, 2340 Beerse, Belgium

[b]Janssen Research & Development L.L.C., 1400 McKean Rd, Spring House, PA 19454

[c]Villapharma Research S.L., Parque Tecnológico de Fuente Álamo. Ctra. El Estrecho-Lobosillo, Km. 2.5- Av. Azul 30320 Fuente Álamo de Murcia, Murcia, Spain

Key words. HBV, TLR7, Quinazoline

Abstract. The discovery of a novel series of highly potent quinazoline TLR 7/8 agonists is described. The synthesis and structure–activity relationship is presented. Structural requirements and optimization of this series toward TLR 7 selectivity afforded the potent agonist **48**. Pharmacokinetic and pharmacodynamic studies highlighted **48** as an orally available endogenous interferon (IFN-α) inducer in mice.

Toll-like receptors (TLR) are expressed on multiple cell types and detect characteristic components common to many pathogens, allowing the host to recognize the first signs of attack by an exogenous organism. Activation of these plasma membrane or endosomal receptors leads to NF-κB/IRF/AP-1 signaling and mobilizes defense mechanisms aimed at eliminating the invading pathogens.[1]

There are 10 TLR paralogues identified to date in humans.[2] Activation of TLRs regulate the expression of inflammatory cytokines/chemokines with or without type I interferons (IFN-α/β), which can lead to the preferential enhancement of innate anti-microbial responses and antigen-specific humoral and cell-mediated immune responses. TLR 7 and 8, expressed in the membranes of endosomes in the cells, recognize single stranded viral RNA and their agonists induce a T_H1-type immune response.[2-4] In humans, TLR 7 is mainly expressed in plasmacytoid dendritic cells (pDC), inducing their activation and production of IFN-α, and to a lower extent on B cells, inducing their proliferation and secretion of antibodies. Conversely, TLR 8 is mainly expressed in monocytes and myeloid DCs (mDCs), inducing a strong T_H1 profile.[5-7] Therefore, TLR 7/8 agonists have been pursued for the treatment of viral infections, tumors, asthma, and as vaccine adjuvants.[8-9] This report focuses on the effort towards a small-molecule, orally available TLR7 selective agonist with a high first pass effect, which limit the cytokine levels in the plasma, and result in an immune response that could be beneficial in the treatment of the above described ailments.

Several chemical series of TLR 7 agonists that induce endogenous IFN-α have been described in the literature (Figure 1). Among them are the imidazoquinoline R837 (**1**),[10] an 8-oxopurine series (e.g. **2**),[11-12] and the pteridinone class of agonists represented by GS-9620 (**3**).[13]

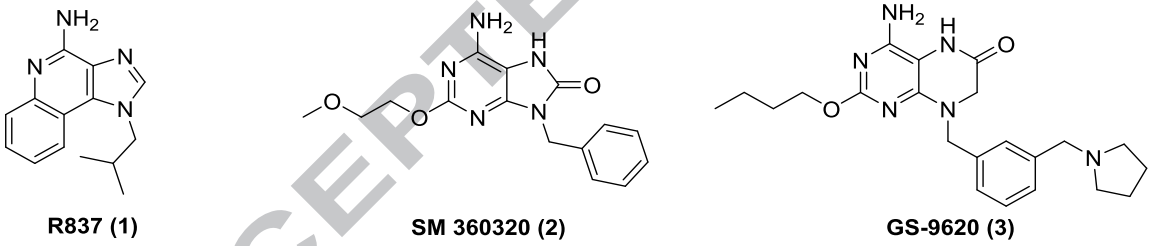
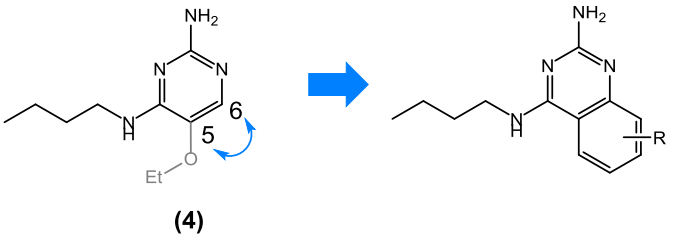


Figure 1. Described TLR 7/8 agonists.

In this manuscript, we report on our efforts leading to a new series of TLR 7 selective agonists which are derived from compound (**4**) which originates from our previous work on dual TLR 7/8 agonists,[14a] and is structurally different from our lead TLR 7 selective series.[14b] While agonists of the TLR 8 receptor may provide advantageous T_H1 response, there may be disadvantageous consequences of proinflammatory cytokine induction.[6] Conceptually, the core structure of **4** was altered with the aim of establishing a new scaffold that would allow us to progress toward TLR 7 selectivity by empirical structure optimization. A fused benzene ring across the 5,6-bond of the pyrimidine of **4** led to a novel series of 2,4-diaminoquinazolines (Figure 2). Literature

search indicated that 2,4-diaminoquinazolines were described as immunomodulators in antiallergy therapy, however their TLR activity was not disclosed.[15] 2-Aminoquinolines and pyrido[2,3-*d*]pyrimidines have also been described as TLR 8 selective agonists.[16,17]

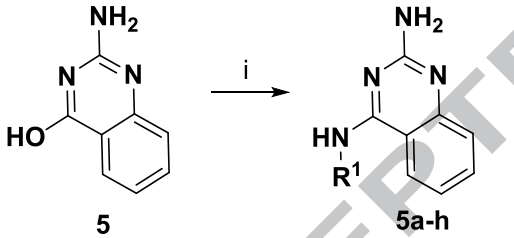


(4)

Figure 2. New series of quinazoline TLR 7/8 agonists.

We considered the quinazoline class as interesting given the advantage that each position of the fused benzene ring can be explored by well described chemistry. Diaminoquinazolines have been described as drug-like molecules such as HSP-90 inhibitors,[18] inhibitors of β-catenin/Tcf-4,[19] and as anti-HIV agents.[20]

The strategy toward TLR 7 selectivity began by examining amine substitution on the unsubstituted scaffold (Table 1). 2-Amino-3*H*-quinazolin-4-one (**5**, Scheme 1) was coupled with a small set of amines via BOP [21] in anhydrous DMF in the presence of DBU to afford compounds **5a-h**.

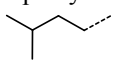
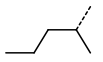
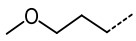


5 5a-h

Scheme 1. Synthesis of compounds **5a-h**: (**i**) DBU, BOP, RNH_2, DMF, rt, 16h.

Table 1

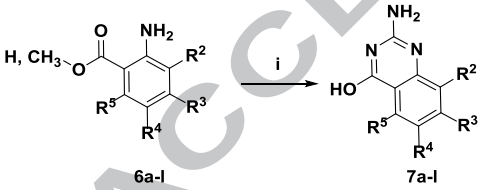
SAR of the amines on the unsubstituted scaffold.

Entry	R^1	Human TLR 7 (LEC µM)	Human TLR 8 (LEC µM)	hPBMC (LEC µM)
4	*n*-butyl	4.7	1.1	1.43
5a	methyl	18.7	6.87	8.88
5b	ethyl	3.07	0.77	2.27
5c	*n*-propyl	1.07	0.93	0.67
5d	*n*-butyl	0.17	0.07	0.03
5e	*n*-pentyl	0.06	0.10	0.04
5f		>25	2.11	1.50
5g		0.71	0.17	0.10
5h		0.46	0.01	0.04

All compounds CC_{50} > 25µM. See supplementary information for assay conditions.

Assessment of TLR agonism was determined in a HEK-293 cell based assay transiently transfected with either TLR 7 or TLR 8. A second assay was employed where compound was incubated with conditioned media from human peripheral blood mononuclear cells (hPBMCs), then the supernatant transferred to assess anti-HCV activity in the replicon system, a confirmation of IFN-α induction (see supporting information). It was found that *n*-butylamine analog **5d** was over 15 times more potent than its congener **4** of the pyrimidine series. Among the selected amines, *n*-butylamine (**5d)** and *n*-pentylamine (**5e**) analogs were most potent on TLR 7 and TLR 8, while smaller alkyl chains showed considerably less agonist potential (**5a, 5b, 5c**). Branching the terminus of the carbon chain with a methyl group as in **5f**, or at the αcarbon (**5g**), resulted in increased TLR 8 selectivity. A similar observation was made when the chain contained an ether as in **5h**.

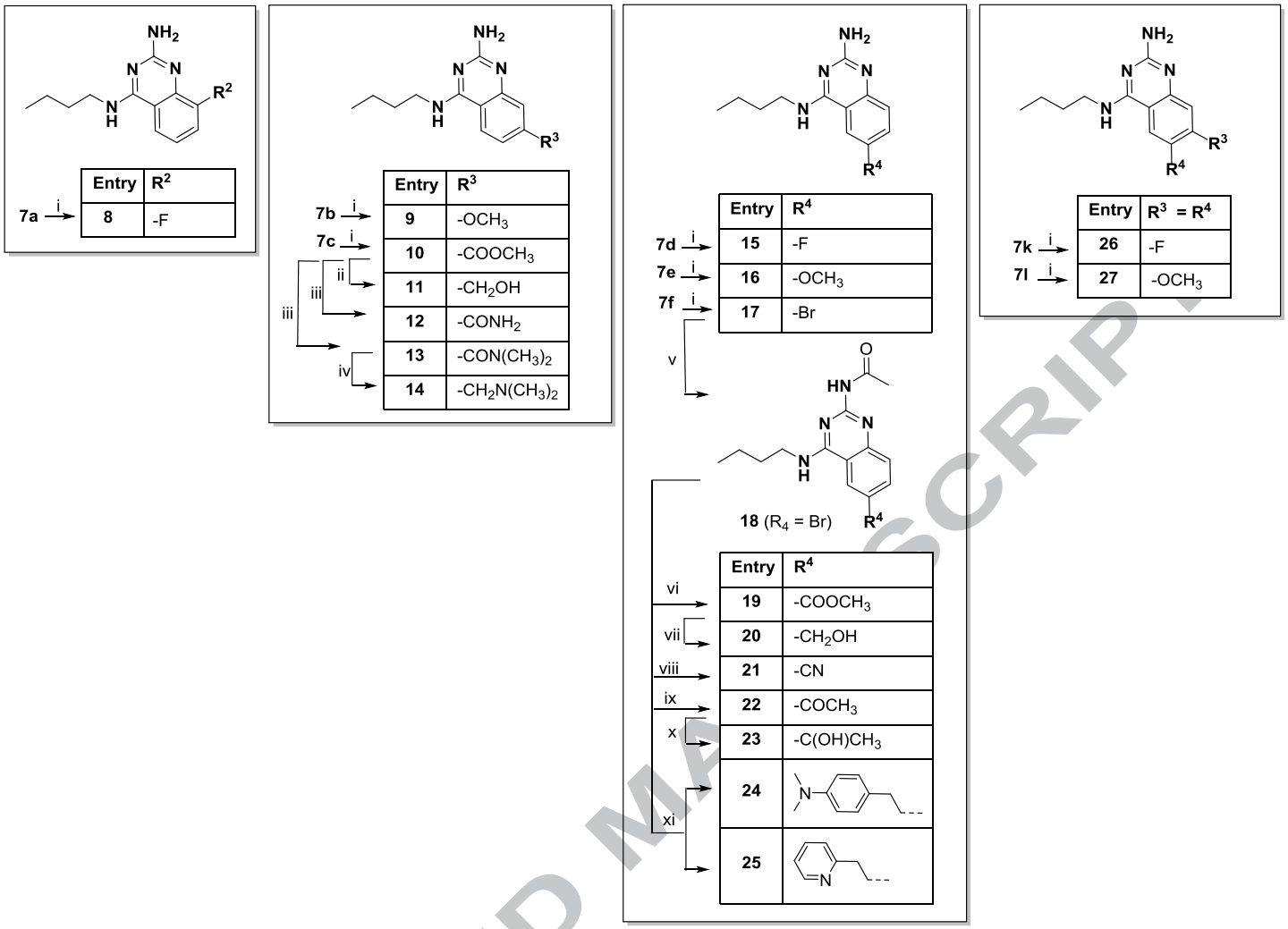
It has been previously described that any change to the 2-amino group leads to loss of activity,[13,14] therefore this group was held constant. Next, the substitution pattern of the fused benzene ring wasassessed. While the *n*-pentylamine analog (**5e**) gave the highest potency (Table 1), it was decided to keep the *n*-butylamine constant for facile comparison with our previous series,[14] as well as other published work,[13, 22] where the *n*-butylamine, and *n*-butoxy analogues retained high activities. The synthesis began with commercially available anthranilic esters or acids which were heated in acidic conditions in the presence of excess cyanamide, using an alcoholic solvent or diglyme, analogous to described methods to afford 2-amino-4-hydroxyquinazolines **7a-l** (Scheme 2, Table 2).[23]



Scheme 2. (**i**) HCl, NH_2CN, EtOH, 100°C, 16h

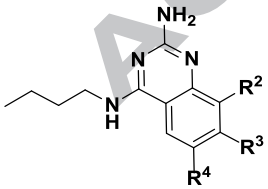
Table 2.
2-amino-4-hydroxyquinazolines

Entry	R^2	R^3	R^4	R^5
7a	F	H	H	H
7b	H	OCH_3	H	H
7c	H	CO_2CH_3	H	H
7d	H	H	F	H
7e	H	H	OCH_3	H
7f	H	H	Br	H
7g	H	H	H	Cl
7h	H	H	H	CH_3
7i	H	H	H	OCH_3
7j	H	H	H	Br
7k	H	OCH_3	OCH_3	H
7l	H	F	F	H

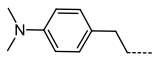
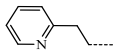


Scheme 3. Synthesis of compounds **8-27:** (**i**) DBU, BOP, *n*-butylamine, DMF, rt, 16h; (**ii**) LAH, THF, -78°C to 0°C, 2h; (**iii**) **1**. LiOH(aq), CH_3OH, THF, rt; **2**. $PO(OEt)_2CN$, amine, NEt_3, DMF, rt, 2h; (**iv**) BH_3-$S(CH_3)_2$, THF, 80°C, 3h; (**v**) Ac_2O, reflux, 1.5h (**vi**) $Pd(OAc)_2$, dppp, KOAc, THF, CH_3OH, 30 bar CO, THF, 120°C, 16h; (**vii**) LAH, THF, -78°C to 0°C, 2h; (**viii**) **1**. $Zn(CN)_2$, $Pd(PPh_3)_4$, DMF, microwave, 160ºC, 10min; **2**. $NaOCH_3$, CH_3OH, 60°C, 1h; (**ix**) **1**. tributyl(1-ethoxyvinyl)tin, $PdCl_2(PPh_3)_2$, DMF, 80ºC, 16 h; **2**. HCl (1N), rt, 2h; **3**. $NaOCH_3$, CH_3OH, 60°C, 1h; (**x**) $NaBH_4$, CH_3OH, rt, 2h; (**xi**) **1**. (Het)arylacetylene, $Pd(PPh_3)_2Cl_2$, $P(Ph)_3$, CuI, Et_2NH, DMF, 80°C, 16 h; **2**. $NaOCH_3$, CH_3OH, 60°C, 1h; **3**. Pd/C 10%, H_2, THF, rt.

Table 3.
SAR of the scaffold substitution.



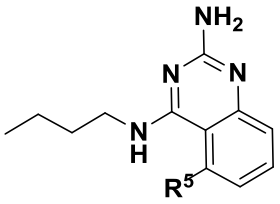
Entry	R^2	R^3	R^4	Human TLR7 (LEC μM)	Human TLR8 (LEC μM)	hPBMC (LEC μM)
5d	H	H	H	0.17	0.07	0.03
8	F	H	H	0.2	0.3	0.07
9	H	OCH_3	H	0.9	0.3	0.15
11	H	CH_2OH	H	0.1	0.1	0.05

12	H		H	0.4	2.0	0.15
13	H		H	0.1	0.5	0.06
14	H		H	0.2	0.04	0.04
15	H	H	F	0.3	0.1	0.11
16	H	H	OCH_3	5.6	1.8	2.44
20	H	H	$-CH_2OH$	0.9	0.2	0.14
21	H	H	-CN	>25	7.1	13.2
22	H	H	$-(CO)CH_3$	6.1	1.9	2.39
23	H	H	$-C(OH)CH_3$	12	5.8	8.49
24	H	H		0.2	0.5	0.15
25	H	H		0.1	0.3	0.04
26	H	F	F	1.8	0.1	0.26
27	H	OCH_3	OCH_3	10	1.1	2.05

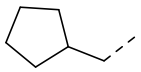
All compounds CC_{50} > 18 μM. See supplementary information for assay conditions. [b]LEC values were averaged when determined in two or more independent experiments.

The 2-amino-4-hydroxyquinazolines (**7a-l**) (Scheme 2, Table 2) were coupled with *n*-butylamine via BOP [21] in anhydrous DMF in the presence of DBU to afford the initial agonists. Table 3 displays the substitution on three positions of the scaffold and further derivatives were made using the aryl bromide or hydroxyl products as intermediates toward new agonist products (*vide infra*). N^4-butyl-8-fluoroquinazoline-2,4-diamine (**8**) was found to have dual activity on TLR 7 and TLR 8, and in the same range as **5d**. At the 7-position of the quinazoline (Table 3, R^3), methoxy (**9**), or hydroxymethyl (**11**) substituted analogs, which were made by reduction of the corresponding ester (**10**) via LAH, provided no TLR 7 selectivity. Hydrolysis of ester **10**, followed by reaction with ammonia or dimethylamine, under standard coupling reaction conditions afforded amides **12** and **13** respectively, which showed a slight increase in TLR 7 selectivity. Selectivity was found to be reversed for the basic amine **14**, made by reduction of **13** with borane dimethyl sulfide, where five-fold selectivity for TLR 8 was observed. The fluorine substituted analog **15** (Table 3, R^4), showed potency comparable to the unsubstituted **5d** and **8.** To improve the yield for Pd catalyzed reactions, toward compounds **19-25**, intermediate **17** was first acylated by refluxing acetic anhydride for 1.5 hours. The congener with the methoxy group (**16**), and alcohol (**20**), made by reduction of ester **19** via LAH in THF, showed more potency and selectivity for TLR 8. When a cyano group (**21**) was introduced by Pd catalyzed cyanation with $Zn(CN)_2$ in DMF via the aryl bromide **18**, activity was greatly reduced. The ketone derivative **22**, derived from **18**, via Stille reaction with tributyl(1-

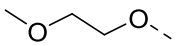
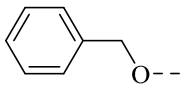
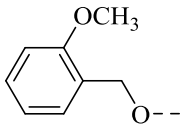
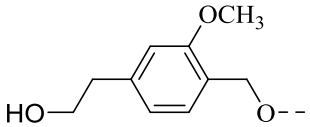
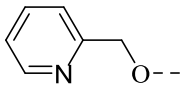
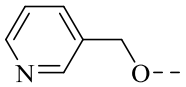
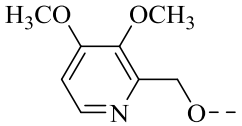
ethoxyvinyl)tin in DMF at 80°C, resulted in an overall reduction in activity. Reduction of the ketone agonist **22** with $NaBH_4$ in methanol led to the corresponding alcohol derivative **23**, which further decreased potency on both TLR 7 and TLR 8. Derivatives **24** and **25** were built via **18**, and the corresponding alkynes, formed in the initial step under standard Sonogashira reaction conditions, followed by catalytic hydrogenation to afford the saturated analogs **24** and **25**. These analogs displayed high potency but unfortunately no TLR 7 selectivity. Finally, disubstituted analogs **26** and **27** (Table 3) showed selectivity for TLR 8 where the difluoro substitution (**26**) was observed to have greater agonist potential compared to the dimethoxy analog (**27**).



7g → i
7h → i
7i → i
7j → i
ii
iii, iv, i
v
vi

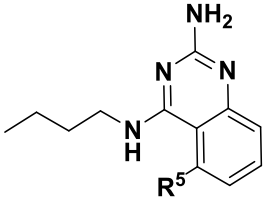
Entry	R^5
28	-Cl
29	$-CH_3$
30	$-OCH_3$
31	-Br
32	
33	
34	-OH

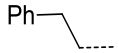
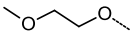
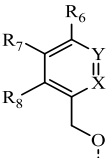
35 to 41

Entry	R^5
35	
36	
37	
38	
39	
40	
41	

Scheme 4. Synthesis of compounds **28-41**: (**i**) DBU, BOP, *n*-butylamine, DMF, rt, 16h; (**ii**) $Pd(dppf)Cl_2$, cyclopentylmethyl-trifluorborate, Cs_2CO_3, water, THF, microwave, 180°C, 1h; (**iii**) phenylacetylene, $Pd(PPh_3)_2Cl_2$, $P(Ph)_3$, CuI, Et_2NH, DMF, 80°C, 16h; (**iv**) Pd/C 10%, H_2, THF, rt; (**v**) pyridine HCl, pyridine, 120°C, 16h; (**vi**) alkylbromide, Cs_2CO_3, DMF, rt, 16h.

Table 4.
SAR of the substituent variations on R^5

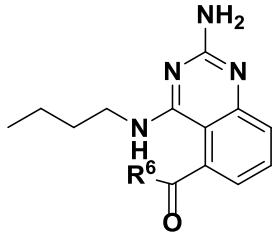


Entry	R^5	R_6	R_7	R_8	X	Y	hTLR7 (LEC μM)	hTLR8 (LEC μM)	hPBMC (LEC μM)	CC_{50} (μM)	Metabolic stability[a](m,h)	
28	Cl						1	0.1	0.17	>25	88	99
29	CH_3						0.5	0.3	0.32	>25	100	96
30	OCH_3						0.4	0.1	0.10	>25	100	98
32							0.12	>25	0.09	>25	nd	nd
33							0.13	0.47	0.27	>25	97	24
35							0.1	0.2	0.12	>25	98	59
36		H	H	H	C	C	0.16	1.66	0.04	10	97	11
37		H	H	OCH_3	C	C	0.27	>25	0.33	>25	98	48
38		CH_2OH	H	OCH_3	C	C	0.03	>25	0.018	16	98	43
39		H	H	H	N	C	0.49	18.5	0.043	>25	99	60
40		H	H	H	C	N	0.03	0.53	0.011	>25	99	44
41		H	OCH_3	OCH_3	N	C	0.03	>25	0.034	16	98	37

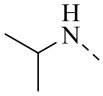
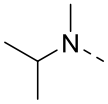
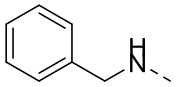
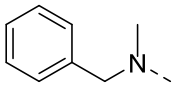
See supplementary information for assay conditions. nd; not determined. [a]Percent metabolized after 15 min. at 1μM in liver microsomes.

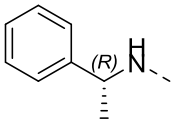
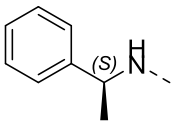
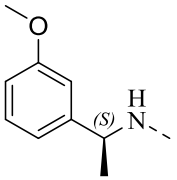
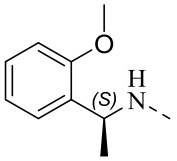
Investigation into the structure−activity relationship of the substituent variation (R^5) positioned ipsilateral to the *n*-butylamine explored the effect on TLR 7 selectivity and the synthesis is described in Scheme 4. Smaller groups such as chlorine (**28**), methyl (**29**), or methoxy (**30**) showed selectivity for TLR 8, whereas the larger cyclopentylmethyl analog **32**, made by reacting the aryl bromide intermediate **31** with cyclopentylmethyl trifluoroborate under modified Suzuki conditions,[24] was potent and selective for TLR 7 (SI > 200 over TLR 8) (Table 4). Phenethyl derivative **33**, obtained by reaction between the bromoquinazoline **7j** and phenylacetylene under standard Sonogashira conditions, followed by reduction of the alkyne via catalytic hydrogenation, showed reduced selectivity. The 2-methoxyethoxy derivative **35** was generated by demethylation of **30** via pyridine hydrochloride, followed by alkylation of the resulting phenolic oxygen of **34** with 1-bromo-2-

methoxyethane in DMF and Cs_2CO_3 as a base. Compounds **32** and **33** implied that TLR 7 selectivity could be achieved with a distal, sterically hindered moiety, placed one or two atoms away from the quinazoline scaffold and adjacent to the butylamine. In view of those results, a small subset of benzyl ethers was prepared (compounds **36-41**). Benzylether analog **36**, synthesized in the same manner as **35**, showed TLR 7 selectivity (SI = 10 over TLR 8) but unfortunately toxicity in the HEK cell line (HEK293 CC_{50} = 10 μM). Potency and selectivity could be further enhanced with the 2-methoxybenzyl group (**37**), hinting at a possible influence of the rotation around this bond, (SI = 90 over TLR 8). To further build on this trend toward TLR 7 selectivity, the (2-methoxy-4-(hydroxymethyl) benzyl congener **38** led to a further increase in potency and concomitant increase in SI > 200 over TLR 8. The pyridine analogs (**39, 40**) demonstrated that TLR 7 selectivity varied with the position of the nitrogen on the aromatic ring, and were overall less selective. Addition of methoxy groups to the pyridine ring, as in **41**, resulted in an enormous boost in TLR 7 selectivity compared to **39**. The activity and selectivity profile of the benzyl ethers **36-41** was attractive, however inhibition of certain CYP450 isozymes (e.g. compound 36: CYP450 1A2 IC_{50} = 0.3 μM, CYP450 2D6 IC_{50} = 5.7 μM) was observed. This was less of a concern since our target product profile consisted of a TLR 7 selective agonist with a high first pass effect. Moreover, literature data suggests that dosing regimens less than once daily may be sufficient.[25] Despite this, further optimization was desired. Exploration in moieties at the carbon adjacent to the *n*-butylamine persisted with the design of carboxylic amides, where variable amines could be installed, allowing the one to two bond distance from the quinazoline scaffold to target optimal activity and TLR 7 selectivity.



7j $\xrightarrow{\text{i, ii}}$ **42 to 50**

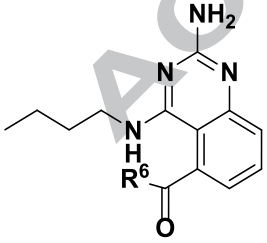
Entry	R^6
42	
43	
44	
45	
46	

Entry	R^6
47	
48	
49	
50	

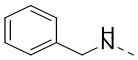
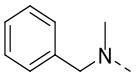
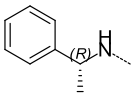
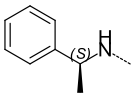
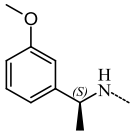
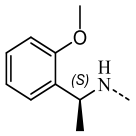
Scheme 5. Synthesis of compounds **42-50**: (**i**) amine, $Pd(OAc)_2$, dppp, KOAc, DIPEA, 50 bar CO, THF, 100°C, 16h; (**ii**) DBU, BOP, *n*-butylamine, DMF, rt, 16h.

Table 5

SAR of the amide variations



Entry	Amines	hTLR7 (LEC µM)	hTLR8 (LEC µM)	hPBMC (LEC µM)	Metabolic stability[a](m,h)	
42		0.35	0.39	0.15	90	23

43		2.05	15.55	0.56	45	14
44		5.62	>25	1.92	nd	nd
45		0.06	1.6	0.03	92	28
46		0.53	>25	0.27	99	98
47		2.29	>25	>25	75	32
48		0.09	>25	0.14	68	31
49		0.37	>25	0.5	96	97
50		0.28	>25	0.14	99	99

All compounds CC_{50} > 25 μM nd; not determined. [a]Percent metabolized after 15 min. at 1μM in liver microsomes.

The carboxylic amide variations were constructed via palladium catalyzed aminocarbonylation in an autoclave by reacting bromoquinazoline **31** with excess amine, and 50 bar CO(g) in THF, as depicted in Scheme 5. Strategically, many amines are commercially available with a range of size, morphology and basicity to rapidly scan for TLR 7 selectivity in parallel. The carboxylic amide variations demonstrated that the methyl amide (**42**), showed potent dual agonist potential, but the more sterically hindered isopropylamide analog **43** decreased activity**.** The *N*-methyl, *N*-isopropyl analog **44**, essentially blocking the amide hydrogen bond donor, demonstrated a further loss in potency. Benzylamide **45** showed intriguing potency but meager TLR 7 selectivity. The *N*-methyl, *N*-benzylamide analog **46** as compared with congener **45**, again implied that the hydrogen bond donor is important for TLR 7 potency. Relocating the methyl group to the alpha-position revealed enantiospecific activity (**47** vs. **48**) where the (*S*)-α-methylbenzyl isomer **48** was clearly superior in

activity (TLR 7 LEC = 90 nM) to the (*R*)-enantiomer **47** and more selective (SI > 200 over TLR 8). In contrast with the previous subseries of benzyl ethers (**36-41**), installation of a methoxy group in this amide subseries afforded agonists of lower potency (**49** and **50**) with respect to **48**. Generally, all modifications afforded metabolically less stable compounds, suitable for the targeted high clearance profile. **48** was 31% metabolized in human liver microsomes compared to 98% for the close analog **46**. To validate these interesting findings, pharmacokinetic studies in rat were conducted, where the dual agonist **5d** was compared with the TLR 7 selective agonists **46**, and the more puissant and metabolically stable **48**. Compound **48** displayed no CYP inhibition up to 10 μM, while **46** possessed some degree of inhibition (e.g. CYP450 3A4 IC_{50} = 8.5 μM, CYP450 2D6 IC_{50} = 5.6 μM). Subsequently, pharmacokinetic and pharmacodynamic evaluation in mice are reported for agonists **5d** and **48**.

Table 6
Rat Pharmacokinetic Parameters.

Entry	T_{max} plasma (h)	C_{max} (ng/mL or g)			AUC_{0-last} (ng·h/mL or g)			%F	Metabolic stability[a]		L/P* ratio
		Plasma Portal	Plasma Systemic	Liver	Plasma Portal	Plasma Systemic	Liver		r	h	
5d	0.5	583	33	2030	1520	56	6450	5.4	95	90	>100
46	0.5	209	9	369	621	37	1064	1.2	99	98	30
48	4	276	87	4930	902	439	15841	20	54	31	36

*L/P Based on AUC(0-last), [a]Percent metabolized after 15 min. at 1μM in liver microsomes.

A pharmacokinetic study in healthy male Sprague-Dawley rats investigated the exposure of **5d**, **46**, and **48** in the systemic circulation following intravenous administration of 2.5 mg/kg and in the portal vein, liver and systemic circulation following an oral administration of 10 mg/kg. High plasma clearance of **5d** and **46** resulted in very short half-life and low exposure in the systemic circulation, where clearance of **5d** (Cl = 163 mL/min/kg) exceeded rat liver blood flow (LBF), and **46** (Cl = 55 mL/min/kg) was 79% LBF, resulting in a very short terminal half-life and low systemic concentrations. The α-methylbenzyl analog **48** showed higher C_{max} and AUC values in the plasma given the same oral dose. **48** showed higher C_{max} (4930 ng/g) and AUC (15, 841 ng.h/g) in the liver, based on the comparison of the oral data of **46** and **48**. The increased systemic exposure and exposure observed before in the liver correlates with improved *in vitro* metabolic stability in rat liver microsomes for compound **48**.

Table 7.
Mouse Pharmacokinetic and Pharmacodynamic Parameters.

Entry	Dose*	T_{max}	C_{max}		AUC0−last		mIFN-α		
		Plasma (h)	Plasma (ng/mL)	Liver (ng/mg)	Plasma (ng.h/mL)	Liver (ng.h/g)	Plasma Fmax (pg/ml)	Liver Fmax (pg/mg)	IFN-α T_{max} (h)
5d	1	0.5	3	207	3	133	315	200	1
48	5	1	142	3227	365	6619	2800	325	4

*oral, mg/kg

A mouse in vivo model was used to demonstrate the initial proof of concept to induce endogenous IFN-α. Single oral administration of 1 mg/kg dose of **5d** and 5 mg/kg dose of the TLR 7 selective and potent **48** were given to healthy C57BL/6 mice. Both compounds were found to be rapidly absorbed given the T_{max} between 0.5 and 1 h and **5d** was found to be rapidly cleared, resulting in a C_{max} of 3 ng/mL, and a negligible exposure (AUC = 3 ng.h/mL) in the plasma. Levels of mIFN-αpeaked at 1h for **5d** and four hours post administration for **48**, in both the plasma and in the liver. **48** showed high levels of mIFN-αsystemically, low systemic and relatively higher liver exposure in mice (liver to plasma ratio = 23), confirming the findings in rat.

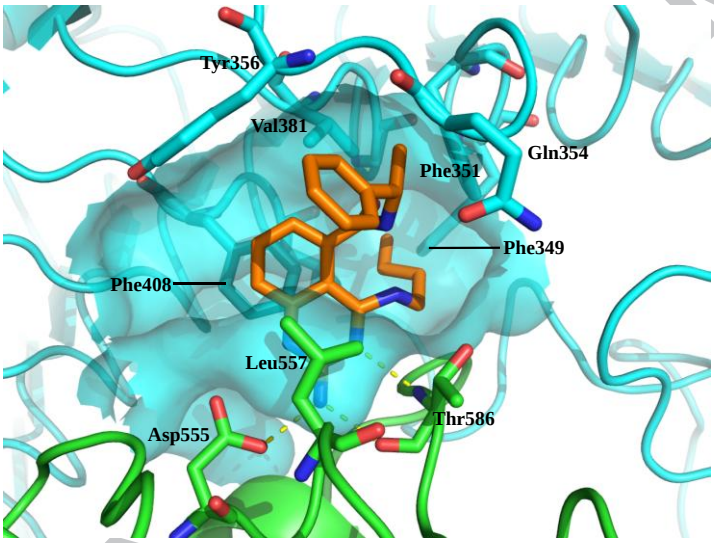


Figure 3. 48, in stick representation, docked into monkey-TLR 7. Monkey-TLR 7 and its dimerization partner are colored in cyan and green, respectively. Hydrogen bonds are shown as yellow, dashed lines.

48 was docked in the Resiquimod binding site identified in the monkey TLR 7 co-crystal structure (PDB 5GMH).[26] This in silico experiment was accomplished using the Glide docking software.[27] The docking poses generated by the protocol were ranked using the GBVI/WSA ΔG scoring function available in MOE.[28] The best

ranked pose of compound **48** was imported, with the monkey TLR 7 protein structure, into a Pymol session to create the Figure 3, which reveals a similar binding mode as described for Resiquimod.[26,29]

The binding of **48** is governed by a network of hydrogen bonds involving the 2-amino group and the nitrogen in the 3-position of the quinazoline scaffold together with the residues Asp555 and Thr586. Our docking study confirmed that the *n*-buylamine moiety fits in the hydrophobic pocket formed by residues Phe349, Phe351 and Val381. The quinazoline scaffold has π-stacking interaction with Phe408 and Leu557, and CH-π interactions with the side chains of those residues. The (*S*)-1-phenethylamine moiety bulges outside of the binding pocket and is conceivably stabilized by amide-π interactions with the backbone between Gln354 and Ile355. An attempt was made to dock **48** into the human TLR 8 structure (PDB 3W3N), yet none of the docking poses obtained showed the expected binding mode nor the expected interactions, especially the *n*-buylamine moiety that did not fit in the hydrophobic sub-pocket formed by Phe349, Phe351, and Val381. This behavior in the docking simulation could explain the poor potency of compound **48** on hTLR 8, which cannot fit properly in the Resiquimod binding site.

In summary, we have described the synthesis and the structure-activity relationship of a series of orally available aminoquinazoline immunomodulators. The pathway toward TLR 7 selectivity was described to include bulky groups ipsilateral to the butylamine group and culminated to the potent and selective (*S*)-2-amino-4-(butylamino)-*N*-(1-phenylethyl)quinazoline-5-carboxamide **48**. Agonist **48** was over 250-fold selective for TLR 7, compared to the reported 30-fold TLR7 selectivity of **3**.[30] The exceptional potency of **48** on TLR7 rivaled that of **2**, and was superior to **1**.[31,32] Low exposure was observed during *in vivo* pharmacokinetic studies in rat and mice after oral administration which is in line with the target profile and expected display low exposure in human despite the slightly higher stability in human liver microsomes. Pharmacodynamic study in mice highlighted **48** as a strong IFN-α inducer after relatively low oral dose. These findings warrant further investigation into the properties of TLR 7 selective immunomodulator **48**.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:....

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33. *Experimental procedure for the synthesis of* **48** Step 1. A 75mL stainless steel autoclave was charged under N_2 atmosphere with **7j** (1 g, 4.12 mmol), (*S*)-(-)-1-phenylethylamine (2.52 g, 20.8 mmol), $Pd(OAc)_2$ (9.4 mg, 0.042 mmol), 1,3 bis(diphenylphosphino)propane (34.4 mg, 0.083 mmol), potassium acetate (818 mg, 8.33 mmol) in THF (15 mL). The autoclave was closed and pressurized to 50 bar CO and the reaction was heated for 16 hours at 100°C. The mixture was cooled and the precipitates were removed by filtration and the filtrate was concentrated to dryness. The residue was partitioned between CH_2Cl_2 and water (set to pH=5 with acetic acid). The organic layer was dried over $MgSO_4$, the solids were removed by filtration and

concentrated to dryness to obtain 2-amino-4-hydroxy-*N*-[(1*S*)-1-phenylethyl]quinazoline-5-carboxamide (789 mg, 61.4% yield). The intermediate was used without further purification in the next step.

Step 2. A 50mL vial was charged with the intermediate (598 mg, 1.94 mmol), anhydrous DMF (15 mL), DBU (1.0 g, 2.68 mmol), and BOP (1.12 g, 2.53 mmol). The mixture stirred at room temperature for 2h, *n*-butylamine (1.34 mL, 13.57 mmol) was added and the reaction stirred at room temperature for 15h. The mixture was concentrated *in vacuo*. A purification was performed via Prep HPLC (Stationary phase: RP XBridge Prep C18 OBD-10µm,30x150mm, Mobile phase: 0.25% NH_4HCO_3 solution in water, CH_3OH), the desired fractions were collected, evaporated to obtain **48** (93 mg, 10% yield) as a solid.

^{1}H NMR (400 MHz, DMSO-d_6) δppm 0.83 (br t, *J*=7.0 Hz, 3 H), 1.16 - 1.31 (m, 4 H), 1.46 (d, *J*=7.0 Hz, 3 H), 3.08 - 3.21 (m, 1 H), 3.25 - 3.33 (m, 1 H), 5.17 (quin, *J*=7.3 Hz, 1 H), 6.10 (s, 2 H), 7.01 (dd, *J*=7.0, 1.3 Hz, 1 H), 7.23 - 7.29 (m, 1 H), 7.31 (dd, *J*=8.5, 1.2 Hz, 1 H), 7.33 - 7.43 (m, 4 H), 7.49 (dd, *J*=8.4, 7.0 Hz, 1 H), 7.54 (t, *J*=4.7 Hz, 1 H), 9.41 (d, *J*=7.9 Hz, 1 H).

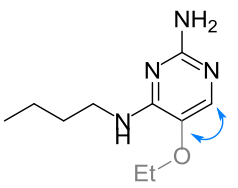
34. *Assessment of TLR7 and TLR8 activity*. The ability of compounds to activate human TLR7 and/or TLR8 was assessed in a cellular reporter assay using HEK293 cells transiently transfected with a TLR7 or TLR8 expression vector and NFκB-luc reporter construct. In one instance, the TLR expression construct expresses the respective wild type sequence or a mutant sequence comprising a deletion in the second leucine-rich repeat (dlRR2) of the TLR. Such mutant TLR proteins have previously been shown to be more susceptible to agonist activation (US 7498409). Briefly, HEK293 cells were grown in culture medium (DMEM supplemented with 10% FCS and 2 mM glutamine). For transfection of cells in 10 cm dishes, cells were detached with Trypsin-EDTA, transfected with a mix of CMV-TLR7 or TLR8 plasmid (750 ng), NFκB-luc plasmid (375 ng) and a transfection reagent and incubated overnight at 37°C in a humidified 5% CO_2 atmosphere. Transfected cells were then detached with Trypsin-EDTA, washed in PBS and resuspended in medium to a density of 1.67 x 105 cells/mL. Thirty microliters of cells were then dispensed into each well in 384-well plates, where 10 µL of compound in 4% DMSO was already present. Following 6 hours incubation at 37°C, 5% CO_2, the luciferase activity was determined by adding 15 µL of Steady Lite Plus substrate (Perkin Elmer) to each well and readout performed on a ViewLux ultraHTS microplate imager (Perkin

Elmer). Dose response curves were generated from measurements performed in quadruplicates. Lowest effective concentrations (LEC) values, defined as the concentration that induces an effect which is at least two-fold above the standard deviation of the assay, were determined for each compound. The Z'-factor of the assay is 0.51 and the %CV of the LEC is between 14 and 50%. Compound toxicity was determined in parallel using a similar dilution series of compound with 30 μL per well of cells transfected with the CMV-TLR7 construct alone (1.67 x 105 cells/mL), in 384-well plates. Cell viability was measured after 6 hours incubation at 37°C, 5% CO_2 by adding 15 μL of ATP lite (Perkin Elmer) per well and reading on a ViewLux ultraHTS microplate imager. Data was reported as CC_{50}.

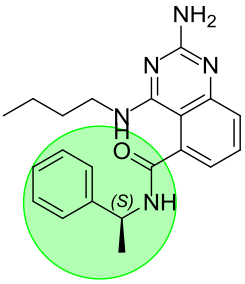
35. *Suppression of HCV replicon replication*. Activation of human TLR7 results in robust production of interferon by plasmacytoid dendritic cells present in human blood. The potential of compounds to induce interferon was evaluated by looking at the antiviral activity in the HCV replicon system upon incubation with conditioned media from peripheral blood mononuclear cells (PBMC). The HCV replicon assay is based on a bicistronic expression construct, as described by Lohmann et al. (Science (1999) 285: 110-113; Journal of Virology (2003) 77: 3007-15 3019) with modifications described by Krieger et al. (Journal of Virology (2001) 75: 4614-4624). The assay utilized the stably transfected cell line Huh-7 luc/neo harboring an RNA encoding a bicistronic expression construct comprising the wild type NS3-NS5B regions of HCV type 1b translated from an Internal Ribosome Entry Site (IRES) from encephalomyocarditis virus (EMCV), preceded by a reporter gene (Firefly-luciferase) and a selectable marker gene (neoR, neomycine phosphotransferase). The construct is flanked by 5' and 3' NTRs (non-translated regions) from HCV type 1b. Continued culture of the replicon cells in the presence of G418 (neoR) is dependent on the replication of the HCV RNA. The stably transfected replicon cells that replicate HCV RNA autonomously and to high levels, encoding inter alia luciferase, were used for profiling of the conditioned cell culture media. Briefly, PBMCs were prepared from buffy coats of at least two donors using a standard Ficoll centrifugation protocol. Isolated PBMCs were resuspended in RPMI medium supplemented with 10% human AB serum and 2 x 105 cells/well were dispensed into 384-well plates containing compounds (70 μL total volume). After overnight incubation, 10 μL of supernatant was transferred to 384-well plates containing 2.2 x 103 replicon cells/well in 30 μL (plated the day before). Following 24 hours of incubation, replication was

measured by assaying luciferase activity using 40 μL/well Steady Lite Plus substrate (Perkin Elmer) and measured with ViewLux ultraHTS microplate imager (Perkin Elmer). The inhibitory activity of each compound on the Huh7-luc/neo cells were reported as EC_{50} values, defined as the compound concentration applied to the PBMCs resulting in a 50% reduction of luciferase activity which in turn indicates the degree of replication of the replicon RNA on transfer of a defined amount of PBMC culture medium. Recombinant interferon α-2a (Roferon-A) was used as a standard control compound. All compounds showed CC_{50} of >24 μM in the HEK 293 TOX assay described above.

36. *Mouse in vivo study*. IFN-αproduction was measured with an ELISA kit from PBL Assay Science. Levels of interferon in plasma and in liver homogenates were assessed. For each timepoint measure, 3 mice were used to calculate the geometric mean of the compound. Studies were performed in AAALAC-accredited sites, and ethical approval by the corresponding ethical committee was obtained.



(**4**) HTS Hit
TLR 7= 4.65 μM
TLR 8= 1.12 μM
TLR 7 selectivity = 0.2



(**48**) TLR 7 selective
TLR 7= 0.09 μM
TLR 8 >25 μM
TLR 7 selectivity >268