Published in final edited form as:

J Med Chem. 2014 March 27; 57(6): 2807–2812. doi:10.1021/jm401356t.

Design, Synthesis, Crystallographic Studies, and Preliminary Biological Appraisal of New Substituted Triazolo[4,3-*b*]pyridazin-8-amine Derivatives as Tankyrase Inhibitors

Paride Liscio^{†,||}, Andrea Carotti[†], Stefania Ascutti[‡], Tobias Karlberg[§], Daniele Bellocchi[†], Laura Llacuna[‡], Antonio Macchiarulo[†], Stuart A Aaronson[‡], Herwig Schöler[§], Roberto Pellicciari^{†,||}, and Emidio Camaioni^{†,*}

[†]Dipartimento di Chimica e Tecnologia del Farmaco, Università degli Studi di Perugia, Via del Liceo 1, 06123 Perugia, Italy

[‡]Mount Sinai School of Medicine, Dept. Oncological Sciences, 1425 Madison Avenue, New York, New York 10029 United States

[§]Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-17177 Stockholm, Sweden

^{||}TES Pharma, via P. Togliatti 22bis, 06073 Località Terrioli, Corciano, Italy

Abstract

Searching for selective tankyrases (TNKSs) inhibitors, a new small series of 6,8-disubstituted triazolo[4,3-*b*]pyridazines has been synthesized and characterized biologically. Structure-based optimization of the starting hit compound NNL (**3**) prompted us to the discovery of 4-(2-(6-methyl-[1,2,4]triazolo[4,3-*b*]pyridazin-8-ylamino)ethyl)-phenol (**12**), a low nanomolar selective TNKSs inhibitor working as NAD isostere as ascertained by crystallographic analysis. Preliminary biological data candidate this new class of derivatives as a powerful pharmacological tools in the unraveling of TNKS implications in physiopathological conditions.

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*Corresponding Author. Phone: +390755855129. Fax: +390755855161. emidio.camaioni@unipg.it.

ASSOCIATED CONTENT

S Supporting Information

Whole experimental data of the synthesis; computational and crystallographic studies; biological tests. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Accession Codes

Coordinates and structure factors of compound **12** in complex with the catalytic domain of TNKS-2 have been deposited to the protein data bank with accession: 4m7b.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

The authors declare no competing financial interest.

INTRODUCTION

Tankyrases (TNKS-1 and TNKS-2) belong to the PARP superfamily. Because of their ability in the transfer of polyADPribose chain (PAR) to targeted proteins, they are also referred to as ADP-ribosyltransferases ARTD-5 and ARTD-6, respectively.¹ TNKS-1 and TNKS-2 share high sequence and structural homology and overlapping functions. In 2009, two independent works reported the first selective TNKSs inhibitors endowed with Wnt pathway disruption properties through axin stabilization. By using a standard TCF/ β -catenin-dependent reporter assay, Huang et al.² identified XAV-939 (**1**, Chart 1) as the first selective TNKSs inhibitor (IC₅₀: TNKS-1, 0.011 μ M; TNKS-2, 0.004 μ M) while by using a similar reporter-based screening approach, Chen et al.³ discovered that structurally distinct small molecules, including IWR-1 (**2**, Chart 1), were equally able to disrupt Wnt signaling via TNKSs inhibition (IC₅₀: TNKS-1, 0.131 μ M; TNKS-2, 0.056 μ M). These two TNKSs inhibitors block Wnt target gene expression stabilizing Axin-1 and -2 proteins by preventing their TNKS-dependent PARsylation and thus promoting β -catenin phosphorylation and degradation. Recently, they have been also cocrystallized with TNKS-2.^{4,5} While **1** (XAV-939) binds in the classical nicotinamide binding site,⁴ **2** (IWR-1) occupies an accessory pocket making interaction with the so-called D-loop.⁵ A thorough review of TNKS inhibitors as well as their pharmacological implications are however reported elsewhere.^{6–8} As a continuation of our research project devoted to the design and synthesis of new inhibitors of the PARP's family,^{9,10} we have recently focused our attention to the discovery of new selective TNKS-1 and TNKS-2 inhibitors.

The Structural Genomics Consortium (SGC) released several crystal structures of the catalytic domain of TNKS-2 in complex with new ligands.^{4,10} Among all new deposited structures, our attention was attracted by the cocrystal of TNKS-2 and *N*-(4-chlorophenethyl)-6-methyl-[1,2,4]triazolo[4,3-*b*]pyridazin-8-amine (NNL, **3**, PDB code 3P0Q).¹⁰ Interestingly, although **3** (NNL) is missing the amide feature, all the interactions formed by the classical PARP inhibitors that bind in the canonical site were conserved (Figure 1S of Supporting Information, (SI)). Herein, with the aim to define structure–activity relationships around this unexplored scaffold, we have synthesized a small library of new triazolopyridazine derivatives bearing different amine in position C-8 with or without a methyl or ethyl group in position C-6. To further investigate the influence of the nitrogen atoms of this heterocycle on the interaction with the enzyme binding site, the scaffold of the most active compound was simplified by the preparation of the corresponding 8-amino-substituted-imidazo-[1,2-*a*]pyridine, -[1,2,4]triazolo[1,5-*a*]pyridine, and -quinoline derivatives, thus reducing the endocyclic nitrogen atoms from 4 to 1. Finally, all the new compounds were tested for their capability to inhibit *in vitro* TNKS-1 and TNKS-2, and the most promising compound was further characterized biologically.

RESULTS AND DISCUSSION

The synthesis of the *s*-triazolo[*b*]pyridazine nucleus was first reported in 1959 by Steck and co-workers.¹¹ Indeed, 8-chlorine-6-alkyl-[1,2,4]triazolo[4,3-*b*]pyridazine derivatives **4** and **5** (Scheme 1) were obtained in high yields following a similar approach of that already reported¹¹ (Scheme 1S, SI). They were then submitted to nucleophilic substitution reactions with suitable amines, thus furnishing the corresponding final compounds **3**, **6–11**, **14–20**, and **22–23** (Scheme 1). Derivatives **11** and **23** bearing a methoxy group in *para*-position of the distal phenyl ring were demethylated by treatment with boron tribromide to obtain the desired hydroxyl derivatives **12** and **24**, respectively, in high yields, while this reaction on *p*-methoxy benzylamino compound **18** afforded the 8-amino-6-methyl-[1,2,4]triazolo[4,3-*b*]pyridazine derivative **21** (Scheme 1).

C-6 unsubstituted derivatives **32** and **33** were prepared following the synthetic procedure depicted in Scheme 2. 3,6-Dichloro-4-pyridazine carboxylic acid **25** was easily synthesized in three steps as previously described^{12,13} (see Scheme 2S, SI). Amino replacement of the carboxyl group of this latter intermediate was accomplished in two steps via Curtius rearrangement of the acid **25** and by subsequent deprotection of the so formed *tert*-butoxy carbonyl amide **26**. Selective exchange of one halogen atom was accomplished by treatment of the dichloro derivative **27**¹⁴ with hydrazine hydrate. 6-Chloro-3-hydrazino-pyridazin-4-ylamine **28**¹⁵ was refluxed in formic acid, affording the key intermediate 6-chloro-[1,2,4]-triazolo[4,3-*b*]pyridazin-8-ylamine **29** in acceptable yields.¹⁶ Removal of the chlorine atom in C-6 position of derivative **29** was affected quantitatively by hydrogenation over a palladium catalyst at 40 psi, furnishing the corresponding [1,2,4]triazolo-[4,3-*b*]pyridazin-8-ylamine **30**.¹⁷ Because of the low reactivity of the previous amine toward acylation reaction, the classical Sandmeyer procedure was applied, converting in high yield compound **30** to its 8-iodo derivative **31**. Nucleophilic substitution of this latter intermediate with 4-methoxyphenethyl amine afforded the compound **32** and, by subsequent demethylation with BBr₃, the compound **33**, in overall good yield. The preparation of other nitrogen-containing heterocycles **40–41**, **45–46**, and **49–50** has been carried out following the synthetic routes reported in Scheme 3.

8-Nitro-1,2,4-triazolo[4,3-*a*]pyridine **36** was prepared in high yield starting from commercially available 2-chloro-3-nitro pyridine **34** as already described.^{18,19} Dimroth rearrangement of intermediate **36** followed by hydrogenation of the nitro group furnished the corresponding isomer 8-amino-1,2,4-triazolo[2,3-*a*]pyridine **38** in overall 60% yield for two steps.¹⁹ Coupling of derivative **38** with 2-(4-methoxyphenyl)acetyl chloride furnished the corresponding amide **39** in 79% yield. The latter amide was easily reduced with lithium aluminum hydride providing the phenethyl amine derivative **40**, which was then submitted to the usual demethylation reaction with boron tribromide, furnishing the desired compound 4-(2-([1,2,4]-triazolo[1,5-*a*]pyridin-8-ylamino)ethyl)phenol **41** in high yield. The synthesis of the *N*-(4-methoxyphenethyl)-*H*-imidazo[1,2-*a*]pyridin-8-amine (**45**) and 4-(2-(*H*-imidazo[1,2-*a*]pyridin-8-ylamino)ethyl)-phenol (**46**) was carried out in a similar procedure of that described before. Indeed, *H*-imidazo[1,2-*a*]pyridin-8-amine (**43**) was synthesized in good yield following a literature protocol^{20,21} and starting from commercially available 2,3-

diaminopyridine (**42**). **43** was then submitted, as previously described, to the acylation reaction with 2-(4-methoxyphenyl)acetyl chloride, providing the corresponding amide **44**, and to subsequent amide reduction, furnishing the phenethylamine derivative **45** in high yield. Finally, demethylation reaction of **45** provided the desired hydroxyl-derivative **46** in 43% yield. Derivatives **49** and **50** were prepared in three steps as already described, starting from commercial 8-aminoquinoline (**47**).

All newly synthesized compounds **3**, **6–24**, **32–33**, **40–41**, **45–46**, and **49–50** were tested for their ability to inhibit in vitro TNKS activity at 1 μ M concentration, and the results are reported in Table 1. One was used as reference compound, showing full inhibition (100%) of both tankyrases at a concentration of 1 μ M. The hit compound **3**, previously cocrystallized with TNKS-2, inhibited at 1 μ M TNKS-1 and -2 enzymatic activities by 75 and 52%, respectively. The corresponding unsubstituted phenethylamino derivative **6** was found to be less active, while the 2-naphthyl analogue **7** was more active, displaying TNKSs inhibition potency of 81 and 74%, respectively. Replacement of the distal phenyl ring with other heterocycles such as 2-thienyl or 2-pyridinyl moieties (**8** and **9**, respectively) gave contrasting results. While the presence of a thiophene decreased the inhibitory activity versus both enzymes (compound **8**, Table 1), the pyridine ring maintained the activity profile only on TNKS-1 (compound **9**). Further substituents were introduced in the *para*-position of the distal phenyl ring in order to modulate the inhibition activity and to increase the water solubility. Electron-donor groups like methoxy, as in derivative **11**, increased the inhibition activity values to 87 and 50% at TNKS-1 and -2, respectively. On the contrary, small electron-withdrawing groups such as fluorine (**10**) did not elicit interesting changes in activity. Replacing the methoxy group with a hydroxy moiety provided derivative **12** that resulted at 1 μ M in full inhibition of TNKS-1 and 82% of inhibition at TNKS-2. Benzoylation of the hydroxy group of derivative **12** as in compound **13** was tolerated, whereas replacement of this hydroxyl with an amine group was found to be ineffective, as derivative **14** was less potent. Replacement of the phenyl ring with *N*-methyl piperazine (**15**) and morpholine (**16**) led to complete loss of the inhibitory activity. The nature of the linker between the distal aromatic moiety and the triazolo[4,3-*b*]pyridazine ring is crucial for the activity vs both TNKSs. Indeed, reducing the linker length to a methylene bridge (**17** and **18**) as well as increasing the spacer length with a propylene group (**19**) afforded to very weak inhibitors. The same trend was observed when the flexibility of the spacer was blocked by introduction of an indane ring as in derivative **20** or when the phenylethyl side chain was removed (**21**). The methyl group in position C-6 (derivatives **11**, **12**) appears to be the optimal substituent as its ethyl replacement or removal, such as in compounds **23–24** and **32–33**, respectively, led to less active species. This was also highlighted by the superposition analysis of our crystal and the 3W51 pdb complex from Larsson et al.,²² where the two methyl groups occupy the same pocket (Figure 2S, SI). The triazolo[4,3-*b*]pyridazine nucleus of compound **12** is crucial for activity, given that simplified analogues **41**, **46**, and **50** were less active (computational studies and Figure 3S, SI). Among all new derivatives, 4-(2-(6-methyl-[1,2,4]triazolo[4,3-*b*]pyridazin-8-ylamino)ethyl)phenol (compound **12**), bearing a *p*-hydroxy phenyl ring in the side chain, was the most potent TNKSs inhibitor of the series and was selected for further biological characterization.

The selectivity of **12** was assessed at a concentration of 10 μM on several members of the PARP superfamily (PARP 1–3, 6–8, 10–12) and compared with AZD2281⁶ (Olaparib), which displayed >70% inhibition in all tested PARPs, as reported in Figure 1 (Table 1S, SI) and in literature.²² At this concentration, **12** fully inhibited both TNKSs whereas it was a weak inhibitor on other PARPs (for further details see Table 1S, SI). Furthermore, as shown in Table 2, **12** exhibited a clean selectivity trend toward PARP-1 and -2 with respect to the reference compound **1**.

Finally, full dose–response curves of **12** against TNKS-1 and -2 were performed with resulting IC_{50} s of 0.012 ± 0.002 and 0.2 ± 0.1 μM , respectively (Table 2; Figure 4S, SI).

Taking into account the excellent potency and the improvement on the selectivity profile, we deemed it appropriate to further investigate compound **12** in cellular assays involving Wnt pathway signaling.

Previous studies have shown that Axin stabilization by TNKS inhibitors can inhibit TCF signaling and, subsequently, cell proliferation of Wnt activated DLD-1 cancer cells.² To assess the effect of our inhibitor **12** on TCF-dependent transcriptional activity, we subjected mass cultures of DLD-1 colorectal cancer cells to a 24 h treatment with increasing concentrations of compound **12** (Figure 2A). The new ligand inhibited dose-dependently TCF reporter. While at low doses (1 μM) reference compounds **1** and **2** displayed high inhibitory activities, at 10 and 20 μM all compounds showed a similar pattern of inhibition. Furthermore, good long-term growth inhibition on DLD-1 was also observed (Figure 2B).

A crystal structure of the TNKS-2 PARP domain in complex with the compound **12** at 1.95 Å resolution was also obtained (Figure 3). Resulting electron density for this ligand was of excellent quality, which allowed for an accurate placement of **12**. The interactions observed for compound **3** mimicking the nicotinamide in NAD^+ were also present for compound **12**. The hydroxyphenylethyl chain is directed toward the solvent and the aromatic ring structure is neatly flanked by hydrophobic side chains (Pro1034, Phe1035, Tyr1050, and Ile1075), whereas the hydroxyl makes interactions, via two waters, with residues on both sides of the pocket.

CONCLUSION

Herein we have synthesized a small library of new [1,2,4]-triazolo[4,3-*b*]pyridazin-8-amine derivatives substituted either in position 6 or 8 as potential selective TNKSs inhibitors. On the basis of the biological results, starting from the selected “hit compound” **3**, a first structure–activity relationship profile was drawn. Thus, the absence of an amide moiety in an anti conformation as the classical pharmacophoric group of PARP inhibitors confers to this new class of derivatives high TNKSs specificity. Compound **12** resulted the most active and selective derivative, endowed with low nanomolar values of IC_{50} on TNKSs proteins and devoid of any PARPs cross-reactivity. Furthermore, it was also assessed as Wnt pathway disruptor. In parallel to this work, Shultz et al.²³ discovered that [1,2,4]-triazol-3-ylamine derivatives are also able to inhibit TNKSs by binding in a similar manner as our molecule. These new classes of compounds are promising isosteres of nicotinamide

derivatives and can be considered new powerful pharmacological tools in the unravelling of TNKS implications in physiopathological conditions.

EXPERIMENTAL SECTION

Chemistry

All procedures are fully described in the SI. All tested compounds were found to have >95% purity determined by HRMS (HPLC/Q-TOF) analyses.

Biology

All PARP assays were done by following the BPS PARP assay kit protocols (BPS Bioscience Inc., San Diego, USA) using human recombinant proteins. To determine TCF-luciferase reporter activity, cells were transduced with TOP TCF reporter lentivirus-expressing firefly luciferase together with renilla luciferase lentivirus (1:20) used to normalize for infection efficiency. Twenty-four h after infection, cells were lysed and analyzed utilizing the dual luciferase reporter assay system. Luciferase reporter activity was calculated by dividing TOP/RL ratio. For colony growth assay, 5×10^3 DLD-1 cells were treated daily with increasing concentrations of compound **1**, **2**, or **12** dissolved in dimethyl sulfoxide. At 10 days, cells were fixed in 10% methanol/acetic acid solution and stained with 1% crystal violet.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATIONS USED

ARTD	ADP-ribosyltransferase
PARP	poly(ADP-ribose) polymerase
TNKS	tankyrase
SGC	structural genomic consortium
PDB	Protein Data Bank

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Figure 1.

Selectivity panel of compound **12** toward several members of the PARP superfamily. Data are from experiments conduct in duplicate at 10 μ M, AZD2281 was used as positive control.

Figure 2.

(A) TOP/RL TCF-luciferase analysis. (B) Cell growth inhibition of DLD-1 colon tumor cells. Compound **12** was compared with standard inhibitors (**1** and **2**) in Wnt activated DLD-1 cells (DMSO is a negative control). Data are mean $\pm$ SEM from at least three independent experiments.

Figure 3.

Crystal structure of **12** with the catalytic domain of TNKS-2. Electron density around **12** displayed as mesh contoured at 1.5 σ (0.39 eÅ⁻³). Hydrogen bonding interactions from **12** to active site residues are highlighted in green. The figure was prepared using PyMOL (<http://www.pymol.org>).

Scheme 1. General Synthesis of 6-Alkyl-[1,2,4]triazolo[4,3-*b*]pyridazine Derivatives^a^aReagents and conditions: (a) $R_2\text{NH}_2$, DMF, 105 °C; (b) BBr_3 , DCM, rt; (c) BzCl , Py, rt.

Scheme 2.

Synthesis of [1,2,4]Triazolo[4,3-*b*]pyridazine Derivatives^a

^aReagents and conditions: (a) DPPA, *t*-BuOH, dioxane, 110 °C; (b) TFA/DCM 1/1, rt; (c) NH₂NH₂·H₂O, 105 °C; (d) CHO₂H, reflux; (e) H₂, Pd/C 10%, DIPEA, EtOH, 40 psi; (f) isoamyl nitrite, CH₂I₂, CH₃CN, reflux; (g) 4-methoxyphenethylamine, DMF, 105 °C; (h) BBr₃, DCM, rt.

Scheme 3.

Synthesis of Other Nitrogen Containing Heterocycles 40–41, 45–46, and 49–50^a

^aReagents and conditions: (a) NH₂NH₂·H₂O, MeOH, rt; (b) CHO₂H, 150 °C; (c) NaHCO₃(ss), rt; (d) H₂, Pd/C 10%, EtOH, 20 psi, 50 °C; (e) 4-methoxyphenylacetyl chloride, Et₃N, DCM; (f) LiAlH₄, Et₂O or DCM, rt; (g) BBr₃, DCM, rt.

Chart 1.
Chemical Structure of Parent TNKSs Inhibitors

Table 1Inhibition Data of Novel Derivatives at 1 μ M Concentration against Recombinant Human TNKS-1 and -2

compd	R_1	R_2	X	Y	% TNKS inhibition ^d	
					1	2
1					100	99
3	Me	(CH ₂) ₂ -Ph- <i>p</i> -Cl			75	52
6	Me	(CH ₂) ₂ -Ph			23	12
7	Me	(CH ₂) ₂ -2-Naph			81	74
8	Me	(CH ₂) ₂ -2-Th			36	24
9	Me	(CH ₂) ₂ -2-Py			72	25
10	Me	(CH ₂) ₂ -Ph- <i>p</i> -F			65	25
11	Me	(CH ₂) ₂ -Ph- <i>p</i> -OCH ₃			87	50
12	Me	(CH ₂) ₂ -Ph- <i>p</i> -OH			100	82
13	Me	(CH ₂) ₂ -Ph- <i>p</i> -OCOPh			63	43
14	Me	(CH ₂) ₂ -Ph- <i>p</i> -NH ₂			45	31
15	Me	(CH ₂) ₂ -4- <i>N</i> -mepiperazine			na	na
16	Me	(CH ₂) ₂ - <i>N</i> -morpholine			na	na
17	Me	CH ₂ -Ph			8	5
18	Me	CH ₂ -Ph- <i>p</i> -OCH ₃			na	na
19	Me	(CH ₂) ₃ -Ph			na	7
20	Me	2-indanyl			16	6
21	Me	H			na	9
22	Et	(CH ₂) ₂ -2-Th			71	33
23	Et	(CH ₂) ₂ -Ph- <i>p</i> -OCH ₃			na	na
24	Et	(CH ₂) ₂ -Ph- <i>p</i> -OH			81	40
32	H	(CH ₂) ₂ -Ph- <i>p</i> -OCH ₃			32	18
33	H	(CH ₂) ₂ -Ph- <i>p</i> -OH			48	33
40		(CH ₂) ₂ -Ph- <i>p</i> -OCH ₃	N	N	na	8
41		(CH ₂) ₂ -Ph- <i>p</i> -OH	N	N	38	20
45		(CH ₂) ₂ -Ph- <i>p</i> -OCH ₃	CH	N	na	na
46		(CH ₂) ₂ -Ph- <i>p</i> -OH	CH	N	8	13
49		(CH ₂) ₂ -Ph- <i>p</i> -OCH ₃			na	na

compd	R ₁	R ₂	X	Y	% TNKS inhibition ^a	
					1	2
50		(CH ₂) ₂ -Ph- <i>p</i> -OH			22	43

^aData are from experiments conduct in duplicate at concentration of 1 μM; na, not active.

Table 2IC₅₀ (μM) and PARP Selectivity of 1 and 12

compd	PARP1	PARP2	TNKS1	TNKS2
1	0.12 ^a	0.046 ^a	0.011 ^a	0.008 ^a
12	>10	>10	0.012	0.2

^aData are from ref 22.