Development of a 1,2,4-Triazole-Based Lead Tankyrase Inhibitor: Part II

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ABSTRACT: Tankyrase 1 and 2 (TNKS1/2) catalyze post-translational modification by poly-ADP-ribosylation of a plethora of target proteins. In this function, TNKS1/2 also impact the WNT/β-catenin and Hippo signaling pathways that are involved in numerous human disease conditions including cancer. Targeting TNKS1/2 with small-molecule inhibitors shows promising potential to modulate the involved pathways, thereby potentiating disease intervention. Based on our 1,2,4-triazole-based lead compound 1 (OM-1700), further structure–activity relationship analyses of East-, South- and West-single-point alterations and hybrids identified compound 24 (OM-153). Compound 24 showed picomolar IC₅₀ inhibition in a cellular (HEK293) WNT/β-catenin signaling reporter assay, no off-target liabilities, overall favorable absorption, distribution, metabolism, and excretion (ADME) properties, and an improved pharmacokinetic profile in mice. Moreover, treatment with compound 24 induced dose-dependent biomarker engagement and reduced cell growth in the colon cancer cell line COLO 320DM.

INTRODUCTION

Tankyrase 1 and tankyrase 2 (TNKS1/2) are members of the poly(ADP-ribose) polymerase (PARP) family of enzymes that regulate the turnover of specific target proteins through covalently linking the cellular redox metabolite NAD⁺ to target proteins in a process called poly-ADP-ribosylation (PARylation). The PAR chain produced in this post-translational modification is subsequently recognized by the E3 ubiquitin ligase ring finger protein 146 (RNF146) leading to polyubiquitination of the PARylated target proteins followed by proteasomal degradation. Independent of their catalytic activity, TNKS1/2 also provides scaffolding functions that are important in the formation of protein complexes. Tankyrase 1 and tankyrase 2 PARylate a plethora of target proteins including peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α), telomeric repeat binding factor 1 (TRF1), phosphatase and tensin homologue (PTEN), AMP-activated protein kinase (AMPK), SRY-box transcription factor 9 (SOX9), and SH3 domain binding protein 2 (SH3BP2). In particular, TNKS1/2 regulate the turnover of AXIN1, AXIN2 (AXIN1/2) and of angiomotin (AMOT) proteins at the crossroad of the elementary wingless-type mammary tumor virus integration site (WNT)/β-catenin and Hippo signaling pathways, respectively. Hence, controlling the catalytic activity of tankyrase by pharmacological intervention provides an attractive tool for reducing WNT/β-catenin and Hippo signaling.

Multiple potent TNKS1/2 inhibiting small molecules, including bicyclic lactams, compounds based on heteroalicyclic amide scaffolds, tricyclic fused ring systems and 1,2,4-triazole scaffolds, have been identified. These compounds block the catalytic domain of TNKS1/2, either by binding with high selectivity to the adenosine binding pocket (which differs from other members of the PARP family) or by binding to the more conserved nicotinamide pocket. The latter binding mode results in a lower selectivity across the PARP family. Other compounds target both pockets in the catalytic domain. Inhibitors based on the 1,2,4-triazole scaffold such as JW74, G007-LK, OD336, and OM-1700 target the adenosine binding pocket of the TNKS1/2 catalytic domain with high selectivity and are therefore able to display selectivity over other members of the PARP family.

Despite the significant progress in developing TNKS1/2 inhibitors, there is currently no viable TNKS1/2 specific...
Inhibitor in clinical practice for any disease indication. Concerns that have hampered clinical trials of TNKS1/2 inhibitors include earlier reports indicating intestinal toxicity,40,41 and bone loss in mouse models,13 although other studies show a beneficial effect of tankyrase inhibition on fracture healing.15 Nevertheless, mice that have been treated for an extended time with a moderate dose of a tankyrase inhibitor have not shown visible adverse effects.43 Ongoing studies suggest that it is possible to overcome a potential biotarget toxicity and recently the tankyrase inhibitors E7449 (a dual inhibitor of PARP1/2 and TNKS1/2) and STP1002 have entered clinical trials with dose escalation studies in patients with advanced solid tumors.44,45 This clearly illustrates the potential of TNKS1/2 inhibitors and justifies the development of drugs directed toward TNKS1/2 inhibition with high potency and an optimized pharmacokinetic (PK) profile.

Here, we further optimize compounds based on the 1,2,4-triazole series as landmark compounds21,28,38,39 and we present compounds reaching picomolar IC_{50} values in a cellular WNT/β-catenin signaling reporter assay, while also showing optimized absorption, distribution, metabolism, excretion (ADME), and pharmacokinetic (PK) properties.

**RESULTS AND DISCUSSION**

**Compound Design Strategy.** Starting from the previously described lead compound 1 (Figure 1)39 and having developed synthetic methodologies for preparing compound iterations in a modular fashion (see Supporting Experimental for the general scheme of synthesis), we aimed at further improving the potency of tankyrase inhibition and optimizing ADME and pharmacokinetic properties of 1. Optimization was motivated by the highly potent compounds discovered during the development of 1 (e.g., compounds 16, 21, and 27 as numbered in our preceding paper39) and we revisited South-, East-, and West-single-point mutations and hybrids thereof. From these structures, we selected six compounds based on their cellular activity and on the diversity of their molecular architecture. The properties of these six shortlisted compounds were evaluated in mouse peroral pharmacokinetic studies and compared with that of compound 1. The best compound of this set was selected and further evaluated with respect to early ADME properties, off-target effects, binding affinity in the catalytic pocket of the TNKS2 protein, as well as inhibition of WNT/β-catenin signaling and proliferation in the colon cancer cell line COLO 320DM. The results from these experiments culminated in the identification of a new 1,2,4-triazole based lead tankyrase inhibitor.

**Structure–Activity Relationship (SAR) Investigation and Biological Evaluation.** To further explore the SAR of the West- and South-regions of 1, single-point modifications departing from 1 were synthesized (Table 1). From these compounds, we concluded that a gain in potency in our in vitro TNKS2 assay and cell-based WNT/β-catenin signaling assay cannot be attained with compounds possessing the East 2-pyridyl group while changing South/West groups, and we therefore focused on compounds with annulated aromatic heterocycles such as naphthyridines and quinoxalines46 (Table 2). Such variations enabled picomolar potencies as these moieties are able to form a significantly more efficient π− π-stacking interaction with His1048 and an additional hydrophobic interaction with Phe1035 as evidenced by the obtained co-crystal structures.39

First, we explored a series based on the East-side 1,5-naphthyridine, either with or without a 3-fluoro substituent (Table 2). Since compound 15a was impaired by a high metabolic instability in mouse microsomes, we aimed at making the structure metabolically more stable by increasing the polarity with the introduction of a South-pyridyl substituion and compounds 16–18 (Table 2) were prepared. In general, more polar compounds are expected to show greater metabolic stability. Compounds with such a South-pyridyl, however, proved to be less potent in the cellular WNT/β-catenin signaling reporter assay compared to 15a and only compound 18b with an IC_{50} value of 6 nM was selected for further characterization (Table 2). To improve potency of compounds 16–18 as earlier observed, a bicyclo[1.1.1]pentane core as an alternative to the cyclobutane influenced activity favorably (see, e.g., compound 19 as numbered in our preceding paper39). Unfortunately, here, compounds built on this bicyclo[1.1.1]pentane core lacked picomolar cellular activity when combined with a South-pyridyl moiety (compounds 19–21, Table 2) and these South-pyridyl structures were abandoned. Furthermore, a South-methyl thioephene group was introduced as a phenyl bio-isostere47 (compounds 22 and 23, Table 2), resulting in potent inhibitors of which compound 22a was selected for further evaluation.

Finally, the introduction of an East quinoxaline moiety instead of the naphthyridine group (compound 24) again led to picomolar cellular activity (IC_{50} = 0.63 nM), and we prepared further variations based on a quinoxaline core to interrogate this part of the pharmacophore. Variations of lead 24 by four fluorine- and methyl-substituted quinoxalines showed similar inhibitory activity (compounds 25–28, Table 3) but by varying the West-moiety, highly efficacious inhibitors could be obtained with the quinoxaline East-group. Three of these compounds were selected for further profiling (compounds 30b, 31a, and 31b). This resulted in a short list of six inhibitors with relatively high structural diversity (Figure 2).

Next, the set of six shortlisted compounds (Figure 2) was subjected to mouse peroral pharmacokinetic (PK) analyses and compared with 1 (Table 4). Here, compounds 22a and 18b displayed a decreased exposure (area under the curve, AUC)
while compound 31a showed an AUC similar to 1. In contrast, compound 30b showed a high AUC, however, with a small volume of distribution (0.33 L/kg), and consequently, none of these compounds were selected for further evaluation.

Further, compounds 31b and 24 showed improved pharmacokinetic properties compared to 1 (Table 4). The AUCs for 31b and 24 were similar and about twice as high compared to the AUC for 1. The peak value ($C_{\text{max}}$) of exposure of 24 was approximately 3 times lower compared to the peak value for compound 31b. In addition, 24 showed a more favorable volume of distribution and solubility compared to these data for compound 31b. Compared to 1, compound 24 possesses an AUC about twice as high and a peak value of about 60% of that of 1 resulting in a higher exposure with a lower peak level. In addition to this, efficacy in the cellular WNT/β-catenin signaling reporter assay was approximately 30 times higher while the clearance of 24 was about half of that of 1.

In summary, based on the overall improved mouse PK profile and the enhanced potency in the cellular WNT/β-catenin signaling reporter assay, compound 24 was designated as the best derivative.

Table 1. West Variations of 1 (OM-1700)^

| ID | Structure | IC$_{50}$ TNKS2 (nM) | IC$_{50}$ HEK293 (nM) |
|----|-----------|----------------------|----------------------|
| 1  | ![Structure](image1.png) | 14 | 19 |
| 2  | ![Structure](image2.png) | 29 | 127 |
| 3  | ![Structure](image3.png) | 9.2 | 12 |
| 4  | ![Structure](image4.png) | 22 | 33 |
| 5  | ![Structure](image5.png) | 16 | 37 |
| 6  | ![Structure](image6.png) | 8.8 | 36 |
| 7  | ![Structure](image7.png) | 8.6 (5.8-13) | 49 |
| 8  | ![Structure](image8.png) | 12 (8.5-16) | 110 |
| 9  | ![Structure](image9.png) | 220 (160-290) | 789 |
| 10 | ![Structure](image10.png) | 93 (28-310) | 355 |
| 11 | ![Structure](image11.png) | 5.2 (2.5-11) | 65 |
| 12 | ![Structure](image12.png) | 13 (6.8-24) | 83 |
| 13 | ![Structure](image13.png) | 4.6 (3.4-6.4) | 37 |
| 14 | ![Structure](image14.png) | 12 (4.9-28) | 27 |

*The IC$_{50}$ values of the compounds of this work were determined with both the TNKS2 biochemical assay (quadruplicates used for each concentration tested, 95% confidence intervals are given in parentheses) and the cellular (HEK293) WNT/β-catenin signaling reporter assay (triplicates used for each concentration tested, T/F-tests were performed for the IC$_{50}$ curve fitting; all $p > 0.95$).*
Subsequently, compound 24 was further characterized and compared to benchmark structure 1 with respect to early ADME properties and off-target effects. Tested ADME parameters such as kinetic solubility, Caco-2 permeability and efflux, microsomal stabilities, mouse plasma stability, and mouse plasma protein binding were found to be in line with 1, while solubility was somewhat lower although in an acceptable range (Table 5). Next, we solved a co-crystal structure of TNKS2-24 demonstrating that 24 binds to the NAD+ cleft of the catalytic domain in a similar manner to the previously reported analogue 1 (Figure 3). The observed electron density is clear for the compound except for an apparently mobile ethoxy group extending toward the nicotinamide site. The previously identified hydrogen bonds of the scaffold were once more observed between 24 and the Tyr1060 and Asp1045 backbones, as well as with a water molecule. The quinoxaline moiety is forming π–π stacking interactions with both His1048 and Phe1035 providing improved binding affinity compared with that of 1.

Finally, to complete the analysis of compound 24, COLO 320DM cells, likely of neuroendocrinal origin, were treated with various doses of 24 to evaluate the efficacy in reducing canonical WNT/β-catenin signaling and the potential as an antiproliferative agent in this cancer cell line. Previously, we have shown that tankyrase inhibition can block WNT/β-catenin signaling and attenuate proliferation and viability in cancer cell lines in vitro and in vivo, including COLO 320DM that is commonly used for testing tankyrase inhibitors. Treatment of COLO 320DM cells with 24 decreased viability with a GI50 value of 10.1 nM and a GI25 value of 2.5 nM (for 1, these values were 650 and 94 nM, respectively), while APC wild-type RKO cells as a control colon cancer line were only modestly affected by treatment with compound 24 (Figure 4a). As previously observed upon tankyrase inhibition, treatment with 24 dose-dependently either increased or decreased the TNKS1/2 protein levels (Figure 4b). Compound 24 also stabilized AXIN1 and AXIN2 proteins and reduced the level of transcriptionally active β-catenin (non-phosphorylated) in both cytoplasmic and nuclear fractions (Figure 4b). In addition, real-time qRT-PCR analyses revealed

| ID | Structure | IC_{50} TNKS2 (nM) | IC_{50} HEK293 (nM) |
|----|----------|------------------|------------------|
| 15a (R=H)| ![Structure](image1) | 4.32 | 0.63 |
| 15b (R=F)| ![Structure](image2) | 3.5-9.5 | 8.5-14 |
| 16a (R=H)| ![Structure](image3) | 5.8 (6.9-9) | 36 |
| 16b (R=F)| ![Structure](image4) | 8.5 (1.2-14) | 8.7 |
| 17a (R=H)| ![Structure](image5) | 4.5 (1.9-11) | 16 |
| 17b (R=F)| ![Structure](image6) | 5.8 (1.3-26) | 9.1 |
| 18a (R=H)| ![Structure](image7) | 5.4 (3.4-8.7) | 13 |
| 18b* (R=F)| ![Structure](image8) | 2.9 (1.9-4.6) | 6.4 |
| 19 (R=H)| ![Structure](image9) | 13 (6.8-25) | 20 |
| 20 (R=H)| ![Structure](image10) | 9.6 (3.9-24) | 30 |
| 21 (R=H)| ![Structure](image11) | 9.4 (4.0-22) | 43 |
| 22a* (R=H)| ![Structure](image12) | 0.73 (0.41-1.3) | 0.81 |
| 22b (R=F)| ![Structure](image13) | 1.6 (1.0-2.6) | 4.1 |
| 23 | ![Structure](image14) | 2.4 (1.6-3.6) | 1.0 |
| 24* (OM-153) | ![Structure](image15) | 2.0* | 0.63* |

*The IC_{50} values of the compounds of this work were determined with both the TNKS2 biochemical assay (quadruplicates used for each concentration tested, 95% confidence intervals are given in parentheses) and the cellular (HEK293) WNT/β-catenin signaling reporter assay (triplicates used for each concentration tested, T/F-tests were performed for the IC_{50} curve fitting; all p > 0.95). * indicates a shortlisted compound. † averages of multiple independent measurements; standard error of the means (SEMs) are shown in Table 5.
reduced levels of transcripts of the WNT/β-catenin signaling target genes AXIN2, DKK1, NKD1, and APCDD1 in a dose-dependent manner (Figure 4c). Collectively, these results showed that 24 both potently and specifically can inhibit WNT/β-catenin signaling activity and block proliferation in COLO 320DM cells.

Table 3. East Quinoxaline Variations

| ID     | Structure | IC_{50} TNKS2 (nM) | IC_{50} HEK293 (nM) |
|--------|-----------|--------------------|---------------------|
| 24* (OM-153) (R^{1}, R^{2}, R^{3} = H) | ![Structure](image.png) | 1.6* | 0.63* |
| 25     | R^{1}=F, R^{2}=H, R^{3}=H | 1.5 (1.0-2.2) | 1.5 |
| 26     | R^{1}=H, R^{2}=Me, R^{3}=H | 1.5 (0.74-3.2) | 2.2 |
| 27     | R^{1}=H, R^{2}=H, R^{3}=Me | 1.1 (0.48-2.5) | 2.4 |
| 28     | R^{1}=H, R^{2}=Me, R^{3}=Me | 2.6 (1.4-4.8) | 4.3 |
| 29a    | ![Structure](image.png) | 1.9 (1.2-2.9) | 1.2 |
| 29b    | ![Structure](image.png) | 2.4 (1.6-3.5) | 0.51 |
| 30a    | ![Structure](image.png) | 1.6 (0.87-3.0) | 0.29 |
| 30b*   | ![Structure](image.png) | 2.0 (1.0-3.7) | 0.80 |
| 31a*   | ![Structure](image.png) | 1.7 (1.1-2.7) | 1.5 |
| 31b*   | ![Structure](image.png) | 1.6 (0.97-2.7) | 0.17 |

The IC_{50} values of the compounds of this work were determined with both the TNKS2 biochemical assay (quadruplicates used for each concentration tested, 95% confidence intervals are given in parentheses) and the cellular (HEK293) WNT/β-catenin signaling reporter assay (triplicates used for each concentration tested, T/F-tests were performed for the IC_{50} curve fitting; all p > 0.95). * indicates shortlisted compound. # averages of multiple independent measurements; SEMs are shown in Table 5.

Figure 2. Short list of six compounds and 1 including their respective biochemical TNKS2 and cellular (HEK293) WNT/β-catenin signaling reporter assays IC_{50} values in nM. clog P and tPSA (in Å²) as calculated by DataWarrior v5.5.0. Moieties in color were different from 1.
CONCLUSIONS

In this further development of our structure-guided lead optimization program of 1,2,4-triazole-based tankyrase inhibitors, we have extensively improved previous lead compound 1 to lead candidate compound 24. We showed that compound 24 possesses improved binding affinity in the catalytic pocket of the TNKS2 protein with concurrently modest selectivity over TNKS1, picomolar IC\textsubscript{50} activity in the cellular WNT/\beta-catenin signaling reporter assay, a clean off-target safety profile, good ADME properties, an optimized mouse PK profile, and potent inhibition of WNT/\beta-catenin signaling and proliferation in COLO 320DM. These results justify testing compound 24 in a pharmacodynamics setting as well as in toxicity models (publication pending).

EXPERIMENTAL SECTION

General Methods. NMR spectra were recorded on a 400 MHz spectrometer with tetramethylsilane as internal standards. Coupling constants are given in hertz. Peaks are reported as singlet (s), doublet (d), triplet (t), quartet (q), septet (hept), multiplet (m), or a combination thereof; br stands for broad.

Liquid chromatography/mass spectroscopy (LC/MS) chromatograms mass spectra were recorded using electrospray ionization (ESI) in positive or negative ionization mode on Agilent 1260 Bin: pump, G1312B, degasser; autosampler; ColCom; DAD G1315C; MSD G6130B ESI; eluent A, acetonitrile; eluent B, 10 mM ammonium bicarbonate in water (base mode) or 0.1% formic acid in water (acid mode). High-resolution mass spectra (HRMS) were recorded with an LC-MS Q Exactive Focus high-resolution mass spectrometer (Thermo Scientific). Calibration was done with the Pierce calibration solutions containing 1-butylamine, caffeine, MRFA, and Ultramark 1621 (positive mode) and the Pierce calibration solution containing sodium dodecyl sulfate, sodium taurocholate, and Ultramark 1621 (negative mode).

Table 4. Mouse PO PK 5 mg/kg and Kinetic Solubility Data of 1 and the Six Shortlisted Compounds

| parameter | compound | \( t_{1/2} \) (h) | \( t_{\text{max}} \) (h) | \( C_{\text{max}} \) (ng/mL) | AUC 0 → \( t \) (ng·h/mL) | AUC 0 → \( \infty \) (ng·h/mL) | MRT 0 → \( \infty \) (h) | \( V_d \) (L/kg) | CL (L/h/kg) | solubility (\( \mu M \)) |
|-----------|----------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|           | 1 (OM-1700)\textsuperscript{a} | 0.67 | 0.25 | 3202 | 2384 | 2388 | 0.69 | 2.03 | 2.09 | >80 |
|           | 18b | 1.00 | 0.25 | 285 | 319 | 322 | 1.56 | 22.4 | 15.6 | >80 |
|           | 22a | 1.17 | 0.25 | 779 | 543 | 547 | 1.39 | 15.5 | 9.14 | 50 |
|           | 24 (OM-153) | 1.50 | 0.5 | 1967 | 4945 | 5038 | 2.39 | 2.15 | 0.99 | 31 |
|           | 30b | 0.69 | 0.5 | 6512 | 15.083 | 15.105 | 1.93 | 0.33 | 0.33 | >80 |
|           | 31a | 0.76 | 0.25 | 2770 | 3404 | 3404 | 1.24 | 1.61 | 1.47 | >80 |
|           | 31b | 0.59 | 0.25 | 5796 | 5313 | 5316 | 1.47 | 0.80 | 0.94 | 13 |

Table 5. Profiling of Compound 24

| parameter | 1 (OM-1700) | 24 (OM-153) |
|-----------|-------------|-------------|
| efficacy | TNKS1 (IC\textsubscript{50} nM (pIC\textsubscript{50} ± SEM))\textsuperscript{a} | 127 (6.90 ± 0.05) | 13 (7.90 ± 0.054) |
|          | TNKS2 (IC\textsubscript{50} nM (pIC\textsubscript{50} ± SEM))\textsuperscript{a} | 14 (7.85 ± 0.04) | 2.0 (8.71 ± 0.069) |
|          | HEK293 reporter assay (IC\textsubscript{50} nM (pIC\textsubscript{50} ± SEM)) | 19 (7.75 ± 0.067) | 0.63 (9.22 ± 0.037) |
|          | COLO 320DM/RKO cells (GI\textsubscript{50}, nM) | 650/>10000 | 10/>10000 |
| ADME | kinetic solubility PBS pH = 7 (\( \mu M \)) | >80 | 31 |
|          | Caco-2 A→B: \( P_{\text{app}} \) (10\textsuperscript{-6} cm/s) | 39.5 | 40.5 |
|          | Caco-2 eflux ratio | 0.61 | 0.64 |
|          | microsomal stability human/mouse/dog CL\textsubscript{int} (\( \mu L/min/mg \) protein) | <5/27/nd | 18/22/3.8 |
|          | mouse plasma stability \( t_{1/2} \) (min) | >120 | >120 |
|          | mouse PPB (%) | 93.92 | 98.58 |
| off-target | PARPs\textsuperscript{b} PARP1/2/3/4/10/12/14/15 (IC\textsubscript{50} \( \mu M \)) | >10 | >10 |
|          | hERG inhibition (IC\textsubscript{50} \( \mu M \)) | >25 | >25 |
|          | Ames test | nongenotoxic | nongenotoxic |
|          | CYP3A4 inhibition (IC\textsubscript{50} \( \mu M \)) | >25 | >25 |
|          | CYP induction (human PXR) | nd | nonactivator\textsuperscript{bc} |
|          | Cerep Safety panel 44 targets@10 \( \mu M \) (inhibition) | clean, (A2A, 53%) | clean, (all <50%) |
| mouse pharmacokinetics | PO PK mouse \( t_{1/2} \) (h) | 0.67 | 1.5 |
|          | PO PK mouse \( C_{\text{max}} \) (ng/mL) | 3202 | 1967 |
|          | PO PK mouse CL (L/h/kg) | 2.09 | 0.99 |
|          | PO PK mouse \( V_d \) (L/kg) | 2.03 | 2.15 |
|          | PO PK mouse AUC 0 → \( t \) (ng/mL) | 2384 | 4945 |
| calculated properties\textsuperscript{d} | MW (g/mol) | 458.5 | 509.6 |
|          | clogP | 3.1 | 3.4 |
|          | tPSA | 95 | 108 |

\textsuperscript{a}See Figure 1, Supporting Information.\textsuperscript{b}See Table 1, Supporting Information.\textsuperscript{c}Highest concentration, 100 \( \mu M \).\textsuperscript{d}Calculated by DataWarrior v5.5.0.
Analysis: 1 μL of a 10 μg/mL sample in MeCN/DMSO 99:1 is injected and data are acquired under full MS mode (resolution 70 000 FWHM at 200 Da) over the mass range m/z of 150 – 2000. Standard ESI conditions compatible with the flow rate are applied: spray voltage, 3.5 kV; auxiliary gas heater temperature, 463 °C; capillary temperature, 280 °C; sheath gas, 58; auxiliary gas, 16; sweep gas, 3; S-lens radio frequency (RF) level, 50. Mass scan range is 150–2000 m/z. Mass resolution is set at 70 000 (<3 ppm mass accuracy). Data are evaluated using Xcalibur Qual Browser version 4.2.47 (Thermo Fisher).

All test compounds were >95% pure by LC/MS and 1H NMR analyses. All spectra as well as preparation of the intermediates and general procedures are given in the Supporting Experiments.

N-(trans-3-(5-(5-Cyclopropoxypyridin-2-yl)-4-(2-fluorophenyl)-4H-1,2,4-triazol-3-yl)cyclobutyl)picolinamide (7). The title compound was prepared according to general procedure F as a white solid (24.3 mg, 75%). LC/MS (ESI) m/z for C26H23N6O2F470 (calculated) 471 ([M + H]+, found). 1H NMR (400 MHz, CDCl3) δ 8.56 – 8.49 (m, 1H), 8.26 – 8.13 (m, 3H), 7.99 (s, 1H), 7.83 (td, J = 7.7, 1.7 Hz, 1H), 7.50 – 7.37 (m, 3H), 7.24 – 7.14 (m, 3H), 4.82 – 4.71 (m, 1H), 3.74 (tt, J = 6.1, 3.1 Hz, 1H), 3.46 (tt, J = 10.3, 5.3 Hz, 1H), 3.11 – 2.96 (m, 2H), 2.52 – 2.35 (m, 2H), 0.84 – 0.71 (m, 4H). HRMS m/z [M + H]+: 471.19393 (calculated), 471.1929 (found), Δ = −2.28 ppm.

N-(trans-3-(4-(2-Fluorophenyl)-5-(5-isopropoxypyridin-2-yl)-4H-1,2,4-triazol-3-yl)cyclobutyl)picolinamide (8). The title compound was prepared according to general procedure F as a white solid (18.3 mg, 77%). LC/MS (ESI) m/z for C26H25N6O2F472 (calculated) 473 ([M + H]+, found). 1H NMR (400 MHz, CDCl3) δ 8.57 – 8.48 (m, 1H), 8.21 (d, J = 6.9 Hz, 1H), 8.18 – 8.08 (m, 2H), 7.91 – 7.79 (m, 2H), 7.48 – 7.38 (m, 2H), 7.25 – 7.14 (m, 4H), 4.76 (h, J = 7.2 Hz,

Figure 3. Co-crystal structure of TNKS2 with 24 (PDB 7O6X). The protein is shown in blue, and 24 in green. The dashed lines in black represent hydrogen bonds, and the red spheres represent water molecules. The σA weighted 2Fo − Fc electron density maps around the ligands are contoured at 1.8σ.

Figure 4. Compound 24 decreased cell growth and inhibited WNT/β-catenin signaling activity in COLO 320DM cells. (a) 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) colorimetric cell growth assay for various doses of 24 in APCmutated COLO 320DM (black) and APCwild-type RKO (gray) cells. After 5 days, the antiproliferative effect of compound treatment was measured at 490 nm. Mean value ± standard deviation (SD) for one representative experiment of more than three repeated assays, each with six replicates, are shown. Dotted lines depict 50% (GI50-value) and 25% (GI25-value) growth inhibition levels and control = 100% (0.1% dimethyl sulfoxide (DMSO)). (b) Representative immunoblots of cytoplasmic TNKS1/2, AXIN1, AXIN2, and cytoplasmic and nuclear transcriptionally active β-catenin (non-phospho) and β-catenin. Actin and lamin B1 show equal protein loading, while # indicates that the same actin immunoblot is used as loading control for both AXIN2 and β-catenin. For (b) and (c), control = 0.001% DMSO. (c) Real-time RT-qPCR analyses of WNT/β-catenin signaling target genes (AXIN2, DKK1, NKD1, and APCDD1). Boxplots show median, first and third quartiles, and maximum and minimum whiskers for combined data from three independent experiments with three replicates each. Dotted lines depict the control mean value = 1. For (a) and (c), analysis of variance (ANOVA) tests (Holm–Sidak method, versus control) are indicated by ***(p < 0.001) and *(p < 0.05), while ANOVA on ranks tests (Dunn’s method, versus control) are indicated by † (p < 0.05).
The title compound was prepared according to general procedure F as a white solid (17.9 mg, 69%). LC/MS (ESI) m/z for C_{24}H_{24}N_{6}O_{2}S 460 ([M + H]^+), found. 1H NMR (400 MHz, CDCl3) δ 8.35 (d, J = 7.2 Hz, 1H), 7.82 (m, 2H), 7.66 (m, 2H), 7.47 (m, 1H), 7.32 (m, 2H), 7.26–7.21 (m, 2H), 7.21–7.13 (m, 1H), 4.83–4.70 (m, 1H), 3.99–3.92 (m, 2H), 3.80 (m, 2H), 3.72 (m, 2H), 1.84 (m, 2H), 1.79 (m, 2H), 1.71–1.72 (m, 1H), 2.12 (m, 2H), 1.34 (m, 2H). 

HRMS m/z [M + H]^+ selected ion monitoring (SIM) = 460.16296 (calculated 460.16200), found Δ = -2.4 ppm.

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HRMS m/z [M + H]^+ selected ion monitoring (SIM) = 460.16296 (calculated 460.16200), found Δ = -2.4 ppm.
The title compound was prepared according to general procedure F as a white solid (12.1 mg, 47%). LC/MS (ESI) m/z for C_{29}H_{26}N_{7}O_{2}S 510 calculated 511 [M+H]^+: 505.2095 (calculated), 505.2086 (found), Δ = -1.87 ppm.

N-(3-(5-(Ethoxy-methyl)-2-furanyl)-4H-1,2,4-triazol-3-yl)cyclobutyl-7-fluoro-1,5-naphthyridine-4-carboxamide (22a).
The title compound was prepared according to general procedure F as a white solid (8.4 mg, 33%). LC/MS (ESI) m/z for C_{26}H_{23}N_{7}O_{2}S 511 (calculated) 512 [M+H]^+: 493.2095 (calculated), 493.2084 (found), Δ = -2.16 ppm.

N-(trans-3-(5-(Ethoxy-2-furyl)-4H-1,2,4-triazol-3-yl)cyclobutyl)-7-fluoro-1,5-naphthyridine-4-carboxamide (18b).
The title compound was prepared according to general procedure F as a white solid (19.2 mg, 73%). LC/MS (ESI) m/z for C_{26}H_{23}N_{7}O_{2}F 510 calculated 511 [M+H]^+: 493.2095 (calculated), 493.2084 (found), Δ = -2.16 ppm.
3.44–3.36 (m, 1H), 2.87 (q, J = 3.8 Hz, 1H), 2.75 (s, 3H), 2.68 (s, 1H), 2.45–2.28 (m, 2H), 1.31 (t, J = 6.9 Hz, 3H). HRMS m/z [M + H]+: 524.22048 (calculated), 524.2198 (found), Δ = −1.3 ppm.

N-(trans-3-(5-(5-Ethoxypyridin-2-yl)-2-(fluorophenyl)-4H-1,2,4-triazol-3-yl)cyclobutyl)-3-dimethylguanin-5-carboxamide (28). The title compound was prepared according to general procedure F as an off-white solid (29.7 mg, 43%). LC/MS (ESI) m/z for C29H21N7OF2 483 (calculated) 484 ([M + H]+, found). 1H NMR (400 MHz, CDCl3) δ 10.68 (d, J = 5.8 Hz, 1H), 8.97 (d, J = 1.8 Hz, 1H), 8.89–8.83 (m, 1H), 8.64 (d, J = 9.6, 3.1 Hz, 1H), 8.26 (dt, J = 8.1, 1.1 Hz, 1H), 8.22 (dd, J = 4.8, 1.6 Hz, 1H), 7.87 (dd, J = 7.8, 3.1 Hz, 1H), 7.67 (td, J = 7.8, 1.8 Hz, 1H), 7.49–7.40 (m, 1H), 7.25–7.14 (m, 4H), 4.84 (ht, J = 7.1, 1.5 Hz, 1H), 3.53 (tt, J = 9.3, 5.3 Hz, 1H), 3.14–3.02 (m, 2H). 2.55 (dd, J = 16.5, 13.1, 9.8, 6.5 Hz, 2H). HRMS m/z [M + H]+: 484.16919 (calculated), 484.16848 (found), Δ = +0.6 ppm.

Biochemical Assay. Recombinantly expressed human tankyrase active constructs for TNKS1 (residues 1030–1317) and TNKS2 (residues 873–1162) and other PARP enzymes used for biochemical assays were produced as previously described.2 The enzymatic assay measures unreacted NAD+ which is chemically reacted into a fluorescent compound. The fluorescent intensity was measured with excitation/emission wavelengths of 372 and 444 nm, respectively, using Tecan Infinity M1000 Pro. New compounds were prepared in half-log dilution series, and the reactions were carried out in quadruplicates with protein and compound controls to exclude the effect of compound autofluorescence. All reactions were performed at ambient temperature. TNKS1 (20 nM) or TNKS2 (5 nM) was incubated for 20 h in assay buffer (50 mM Bis-Tris propane (BTP), pH 7.0, 0.5 mM Tris(2-carboxyethyl)phosphine (TCEP), 0.01% Triton X-100) with compound and 10 μM or 500 nM NAD+, respectively.

The assay conditions for the other PARP enzymes were used as previously described. Reference compound OD336 behaved in this assay as previously reported.2 For single IC50 curves, the 95% confidence interval (asymptotic) was calculated in GraphPad Prism 8.

WNT/β-Catenin Signaling Reporter Assay. The luciferase based WNT/β-catenin signaling pathway reporter assay in human HEK293 cells was performed in triplicates as previously described2 also taking OD336 as a reference compound, which behaved in this assay as previously described.2

ADME. Kinetic solubility, micromosial stability, and plasma stability were determined following our internal protocol (Symere). The Caco-2 and PBB determinations were performed by Cyprotec.

Off-Target. A safety panel (Cerep) of 44 selected targets (n = 2) including hERG Inhibition using 10 μM 24 was performed by Eurofins, Ames and CYP3A4 inhibition assays were performed by Cyprotec, and the PXR assay (CYP induction) was performed at Admesco. The PARP assays are described below.

Mouse Pharmacokinetic Analysis. The pharmacokinetic analyses in mice were performed according to the standard protocols of Medicilon as previously described following approval by local animal experiment authorities (Shanghai, China) and in compliance with FELASA guidelines and recommendations. Three ICR mice were used per treatment group: 5 mg/kg 24 in 5% DMSO, 50% PEG400 (both Sigma-Aldrich), and 45% saline as vehicle. Samples were collected after 5, 15, and 30 min and 1, 2, 4, 8, and 24 h.

Crystallography. The co-crystallization of human TNKS2 catalytic domain (residues 952–1161) in complex with 24 was done in the presence of chymotrypsin (1:100) and based on crystallization efforts previously described.39 Protein (5.3 mg/mL) was mixed with 0.4 mM compound from a 10 mM stock solution in DMSO. The droplets for crystallization were set up using the sitting-drop vapor diffusion method by mixing 200 nL of protein with 100 nL of precipitant solution (0.1 M Bisine, pH 8.5–9.0, 7.5–25% PEG6000). All steps were performed at room temperature. Rod-shaped crystals appeared within 24 h and were cryo-protected using the precipitant solution containing 25% PEG6000 and 20% glycerol.
Data were collected at Diamond Light Source on beamline I04. Diffraction data were processed using the XDS package. The substructure was solved using molecular replacement with Phaser using the structure of TNKS2 (PDB code: SNOB) as starting model. Model building and refinement was done using Coot and Refmac5 respectively. Preparation of the crystal structure images was done with The PyMOL Molecular Graphics System (PyMOL, version 1.8.4.0).

**Proliferation Assay.** Antiproliferative assays using colon cancer cell lines COLO 320DM and RKO were performed as previously described.39,50

**Western Blot Analysis.** Western blot analysis of nuclear and cytoplasmic lysates from compound-treated COLO 320DM cells was performed as previously described.39,50

**RNA Isolation and Real-Time qRT-PCR.** RNA isolation and real-time qRT-PCR were performed as previously described.39,50

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c01264.

Molecular formula strings and data (CSV) General and specific synthetic procedures and spectra for all compounds and inhibition data and crystallography; atomic coordinates and structure factors have been deposited to the Protein Data Bank under accession number 7O6X, and raw diffraction images are available at IDA (https://doi.org/10.23729/4d48eb0-c6e4b7d-a557-3758f5f68d47). The authors will release the atomic coordinates upon article publication (PDF)

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R.G.G.L. and S.A.B. contributed equally to this article, while J.W. and S.K. contributed equally as co-senior authors of this article. The manuscript was written through contributions of all authors, and all authors have given approval to the final version of the manuscript.

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#### Notes

The authors declare the following competing financial interest(s): J.W., M.N., L.L., A.W., R.G.G.L., and S.K. hold patents related to tankyrase inhibitor therapy, and these authors declare no additional interests. The remaining authors declare no competing interests.

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**ABBREVIATIONS USED**

ADME, absorption, distribution, metabolism, and excretion; ADP, adenosine 5'-diphosphate; AKT, serine/threonine kinase; AMOT, angiomin; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; APC, adenomatous polyposis coli; APCDD1, APC downregulated 1; AUC, area under the curve; AXIN, axis inhibition protein; CL, clearance; CMC, chemistry, manufacturing, and control; DKK1, dickkopf WNT signaling pathway inhibitor 1; DMSO, dimethyl sulfoxide; HEK293, human embryonic kidney 293; hERG, human ether-a-go-go-related gene; LC/MS, liquid chromatography/mass spectroscopy; MTS, 3-(4,5-dimethylthiazol-2-yl)-2,5-(3-carboxymethoxyphenyl)-(4-sulfophenyl)-2H-tetrazo- lium; NKDI, NKD inhibitor of WNT signaling pathway 1; NAD, nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; PARP, poly-ADP-ribose polymerase; PARyllate, poly(ADP-ribose)sylate; PGC-1β, peroxisome proliferator-activated receptor-γ coactivator 1 α; PK, pharmacokinetics; PO, per oral; PPB, plasma protein binding; PTEN, phosphatase and tensin homolog; RT-qPCR, reverse transcription quantitative polymerase chain reaction; RNF146, ring phosphatase and tensin homolog; RTF, telomeric repeat factor; tPSA, total polar surface area; TRF, telomeric repeat factor; Vdp, volume of distribution; WNT, wingless-type mammary tumor virus integration site

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