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Identification of Novel Target for Osteosarcoma by Network Analysis

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Background: Osteosarcoma (OS) is a highly complicated bone cancer involving imbalance of signaling transduction networks in cells. Development of new anti-osteosarcoma drugs is very challenging, mainly due to lack of known key targets.


Material/Method: In this study, we attempted to reveal more promising targets for drug design by "Target-Pathway" network analysis, providing the new therapeutic strategy of osteosarcoma. The potential targets used for the treatment of OS were selected from 4 different sources: DrugBank, TCRD database, dbDEMOC database, and recent scientific literature papers. Cytoscape was used for the establishment of the "Target-Pathway" network.

Results: The obtained results suggest that tankyrase 2 (TNKS2) might be a very good potential protein target for the treatment of osteosarcoma. An *in vitro* MTT assay proved that it is an available option against OS by targeting the TNKS2 protein. Subsequently, cell cycle and apoptosis assay by flow cytometry showed the TNKS2 inhibitor can obviously induce cell cycle arrest, apoptosis, and mitotic cell death.

Conclusions: Tankyrase 2 (TNKS2), a member of the multifunctional poly(ADP-ribose) polymerases (PARPs), could be a very useful protein target for the treatment of osteosarcoma.

MeSH Keywords: **Database • Enzyme Inhibitors • MicroRNAs • Osteosarcoma**

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Background

Osteosarcoma (OS) frequently occurs in teenagers and young adults and causes 20% of all primary bone cancers [1]. Despite the success of neoadjuvant chemotherapy followed by surgical resection for osteosarcoma, the survival rate of pediatric patients is still low, and their progression-free survival was reported to be about 23 months [2]. Therefore, discovery of potential diagnostic and effective therapeutic targets used for the treatment of OS is an urgent issue for the development of new OS drugs.

There are dozens of antineoplastic drugs being tested in hundreds of clinical trials for the treatment of osteosarcoma retrieved from the clinicaltrials.gov website [3]. Most clinical candidates involve several crucial cell signaling pathways, particularly protein kinase inhibitors [4]. In addition, the drugs that interacted with DNA, referred to as cytotoxic agents or specific ribozyme inhibitors, are also thought to account for a large proportion in these osteosarcoma clinical trials. Thus, we collected a series of antitumor targets obtained from corresponding experimental drugs for the treatment of OS, as well as the potential therapeutic targets of these drugs for the first step in this study.

MicroRNAs (miRNAs) are small noncoding regulatory RNAs 22–25 nucleotides in length, which widely participate in a number of biological processes [5]. The miRNAs dysfunction that ties many pathological conditions together involves obvious change in several biological processes, including cell proliferation, apoptosis, cell cycle, migration, and invasion [6]. Although this imbalance of miRNAs expression level cannot be considered as the primary cause for various diseases, the expression profiles analysis of miRNAs on a genome-wide scale could contribute to the discovery of new targets, especially for complicated tumor diseases. For example, various recently reported miRNAs suppressed OS cell proliferation, cell cycle progression, and apoptosis, such as miR-20a [7], miR-26a [8], miR-100 [4], miR-124 [9], miR-126 [10], miR-195 [11], miR-205 [12], and miR-491 [13]. Based on these findings, we searched for dozens of various potential targets of OS by querying the validated targets from Tarbase [14], a miRNAs target database.

Although many possible targets used for OS therapy had been reported [15] and many bioinformatics databases such as the Target Central Resource Database (TCRD) have been used to uncover possible targets of OS [16], the most promising targets for clinical use remain to be discovered. In the present study, we explored novel effective targets of OS by network analysis focusing on potential targets collected in as many different ways as possible, and subsequently investigated the underlying mechanism by use of MTT assay and flow cytometry.

Our results may lay the foundation for development of new OS drugs.

Material and Methods

Cell culture

The 4 human OS cell lines – LM7, SaOS-2, U2OS, and MG-63 – were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Beyotime Biotechnology, Shanghai, China). These 4 cancer cell lines were then incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Network construction

The potential targets used for the treatment of OS were selected from 4 different sources: (1) The possible targets retrieved from DrugBank [17] by searching for 28 clinical drugs used for OS (Table 1); (2) The possible kinase targets as shown in Table 2 retrieved from the TCRD database [16] (<https://pharos.nih.gov/idg/index>); (3) The possible targets obtained from Tarbase database on the basis of microRNAs involved with OS validated by the dbDEMOC 2.0 database [18] (Table 3); and (4) The possible targets obtained from Tarbase database on the basis of microRNAs involved with OS validated by recent papers (Table 4). In addition, these targets listed above were imported into the Biological Information Annotation Databases for the molecular function and biological process annotation. To facilitate scientific interpretation of complex relationships between OS targets and signaling pathways, network analysis was performed. The "Target-Pathway" network was generated by Cytoscape (<http://www.cytoscape.org/>) [19], which is an open-source software for visualizing complex networks and integrating these with any type of attribute data.

The MTT assay

Compound NVP-TNKS656 was purchased from SelleckChem. The anti-proliferation activities of prepared compounds were evaluated as described in previous reports [20]. The 4 OS cells – LM7, SaOS-2, U2OS, and MG-63 (5000 cells/well) – were seeded in a 96-well plate, and then incubated with compound (0.5 μM) for 0, 12, 24, or 36 h. Subsequently, 10 μL MTT solution (5 mg/mL; Beyotime Institute of Biotechnology, Haimen, China) was added to each well, followed by incubation for another 4 h. After the supernatant was removed, 100 μL dimethyl sulfoxide was added to each well. The absorbance was detected at 570 nm with a microplate reader.

Table 1. The main clinical drugs that exclude cytotoxic agents used for the treatment of osteosarcoma and their important targets at the clinical Phase I, II, III, and IV currently.

No.	Drugs	Targets at the DrugBank database	
		Name	Gene
1	Zoledronic acid	Farnesyl pyrophosphate synthase	FDPS
		Geranylgeranyl pyrophosphate synthase	GGPS1
2	Leucovorin	Thymidylate synthase	TYMS
3	Afatinib	Vascular endothelial growth factor receptor-2	KDR
4	Rapamycin	Mammalian target of rapamycin	MTOR
5	Sorafenib	Serine/threonine-protein kinase B-raf	BRAF
		Vascular endothelial growth factor receptor 2	KDR
		Mast/stem cell growth factor receptor Kit	KIT
6	Methotrexate	Dihydrofolate reductase	DHFR
7	Pembrolizumab	Programmed cell death protein 1	PDCD1
8	Etoposide	DNA topoisomerase 2-alpha	TOP2A
9	Avelumab	programmed death-ligand 1	PDCD1L1
10	Eribulin	Tubulin beta-1 chain	TUBB1
11	Pazopanib	Vascular endothelial growth factor receptor 1	Flt1
		Vascular endothelial growth factor receptor 2	KDR
		Platelet-derived growth factor receptor alpha	PDGFRA
		Platelet-derived growth factor receptor beta	PDGFRB
12	Lenvatinib	Vascular endothelial growth factor receptor 1	Flt1
		Vascular endothelial growth factor receptor 2	KDR
		Vascular endothelial growth factor receptor 3	FLT4
		Fibroblast growth factor receptor 1	FGFR1
		Fibroblast growth factor receptor 2	FGFR2
		Fibroblast growth factor receptor 3	FGFR3
		Fibroblast growth factor receptor 4	FGFR4
13	Cabozantinib	Hepatocyte growth factor receptor	MET
		Vascular endothelial growth factor receptor 2	KDR
		Proto-oncogene tyrosine-protein kinase receptor Ret	RET
14	Regorafenib	More than 15 protein kinases	—*
15	Nivolumab	Programmed cell death protein 1	PDCD1
16	Paclitaxel	Apoptosis regulator Bcl-2	BCL2
		Tubulin beta-1 chain	TUBB1
17	Lexatumumab	Death receptor 5	TNFRSF10B
18	Irinotecan	DNA topoisomerase 1	TOP1

Table 1. The main clinical drugs that exclude cytotoxic agents used for the treatment of osteosarcoma and their important targets at the clinical Phase I, II, III, and IV currently.

No.	Drugs	Targets at the DrugBank database	
		Name	Gene
19	Gefitinib	Epidermal growth factor receptor	EGFR
20	RO4929097	Gamma secretase	APH1A/B
21	Larotrectinib	Tropomyosin kinase	Trk
22	Ipilimumab	Cytotoxic T-lymphocyte protein 4	CTLA4
23	Tanespimycin	Heat shock protein 90	HSP90
24	Erlotinib	Epidermal growth factor receptor	EGFR
25	Alvocidib	Cyclin-dependent kinase	CDK
26	Cixutumumab	Insulin-like growth factor 1 receptor	IGF1R
27	Romidepsin	Histone deacetylase 1	HDAC1
		Histone deacetylase 2	HDAC2
28	Abemaciclib	Cyclin-dependent kinase 4	CDK4
		Cyclin-dependent kinase 6	CDK6

* Means too many kinases to list in this table.

Table 2. The main potential kinase targets retrieved from the Target Central Resource Database for the treatment of osteosarcoma at the Tclin* and Tchem** development/druggability levels.

No.	Name	Gene
1	Cyclin-dependent kinase 11B	CDK11B
2	Serine/threonine-protein kinase PAK 5	PAK5
3	Dual specificity tyrosine-phosphorylation-regulated kinase 1B	DYRK1B
4	Casein kinase II subunit alpha'	CSNK2A2
5	Membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase	PKMYT1
6	Phosphorylase b kinase regulatory subunit alpha, skeletal muscle isoform	PHKA1
7	Cyclin-dependent kinase-like 3	CDKL3
8	Cyclin-dependent kinase 17	CDK17
9	Cyclin-dependent kinase-like 1	CDKL1
10	Serine/threonine-protein kinase Nek5	NEK5
11	SRSF protein kinase 3	SRPK3
12	Serine/threonine-protein kinase RIO1	RIOK1
13	Phosphorylase b kinase gamma catalytic chain, liver/testis isoform	PHKG2
14	Atypical kinase COQ8B, mitochondrial	COQ8B
15	Phosphorylase b kinase gamma catalytic chain, skeletal muscle/heart isoform	PHKG1
16	Phosphatidylinositol 5-phosphate 4-kinase type-2 gamma	PIP4K2C

Table 2. The main potential kinase targets retrieved from the Target Central Resource Database for the treatment of osteosarcoma at the Tclin* and Tchem** development/druggability levels.

No.	Name	Gene
17	Dual specificity protein kinase CLK4	CLK4
18	Serine/threonine-protein kinase 17A	STK17A
19	Calcium/calmodulin-dependent protein kinase type 1D	CAMK1D
20	Cyclin-dependent kinase 14	CDK14
21	Serine/threonine-protein kinase PRP4 homolog	PRPF4B
22	Microtubule-associated serine/threonine-protein kinase 2	MAST2
23	MAP kinase-interacting serine/threonine-protein kinase 2	MKNK2
24	Serine/threonine-protein kinase tousled-like 2	TLK2
25	Dual specificity protein kinase CLK3	CLK3
26	Dual specificity tyrosine-phosphorylation-regulated kinase 2	DYRK2
27	Thymidine kinase 2, mitochondrial	TK2
28	Serine/threonine-protein kinase Nek7	NEK7
29	Phosphatidylinositol 4-kinase alpha	PI4KA
30	cAMP-dependent protein kinase catalytic subunit beta	PRKACB
31	Protein kinase C theta type	PRKCQ

* These targets have activities in DrugCentral database (approved drugs) with known mechanism of action; ** these targets have activities in ChEMBL or DrugCentral.

Table 3. The key microRNAs with downregulated expression in osteosarcoma samples from the dbDEMC 2.0 database.

No.	microRNAs	Targets form Tarbase (Pred.Score ≥0.9 & Gene name)
1	miR-1	TNKS2, SPRED1, SRSF1
2	miR-126	BRWD3, JARID2, SCD, NPAS2, HIPK2
3	miR-133a	NUP160, SGPP1, COL8A1, FAM160B1, CELF1, TSPAN18, MAP3K2
4	miR-133b	RB1CC1, CELF1, FTL, MCL1
5	miR-142-3p	TCEB3, TEX2, KDELR2, IRAK1
6	miR-144	SNTB2, EIF4G2, ZNF367, ZNF800, CEP350, DCP2, TNKS2
7	miR-150	MTCH2, LDLR, PERP
8	miR-195	CCNE1, WEE1, FBXW7, IPO7, E2F3, SKI, AGO1, SON, CDC27, CDC42SE2, NUFIP2
9	miR-205-5p	CCNJ, CBX1, SNW1
10	miR-206	GJA1, TNKS2, SRSF9, MMD, GPD2, EFN2, SERP1, IFT52, TWF1, ZNF264, PGD
11	miR-223	PARP1, SCARB1, RASGRP1
12	miR-451	PFAS
13	miR-486-5p	ST6GALNAC6, ZNF367
14	miR-497	RAF1, RUNX2, IGF1R, MAP2K1

Table 4. The microRNAs reported for the inhibitory activities against osteosarcoma in these recent papers.

No.	microRNAs	Targets form Tarbase (Pred.Score ≥ 0.9 & Gene name)
1	miR-20a [7]	GINM1, RUFY2, C2CD2, TNKS2, PTPN4, SIK1, EFCAB14, RASL11B, ZNF367
2	miR-26a [8]	ZBTB18, TOB1, REEP3, MSMO1, OSBPL11, DDX3X, NXPE3, HOXA9, ZFHX4, PDHX, EIF4G2
3	miR-100 [4]	NONE
4	miR-124 [9]	VAMP3, CD164, RAB10, TARBP1, HIPK3, LAMC1, RRAGD, PTBP1, SLC35F5, QSER1, DCAF16, STK35, CGN, AGO1
5	miR-125a [38]	MFHAS1, SEMA4C, RORA, FAM118A, KPNA6, ZC3H7B, CDK16
6	miR-126 [10]	BRWD3, JARID2, SCD, NPAS2, HIPK2
7	miR-195 [11]	CCNE1, WEE1, FBXW7, IPO7, E2F3, SKI, AGO1, SON, CDC27, CDC42SE2, NUFIP2
8	miR-205 [12]	CCNJ, CBX1, SNW1
9	miR-216a [39]	GATAD2B
10	miR-382 [40]	NONE
11	miR-491 [13]	GATAD2B, DIRAS1, ANKRD52, IGF2BP1

Apoptosis analysis

MG-63 cells were seeded in 12-well plates, at 1×10^5 cells/well, and incubated for 12 h. Cells were treated with this compound at 4 different concentrations (10, 50, 100, and 200 nM) for 24 h. The cell apoptosis was detected using an Annexin-V-FITC Apoptosis Detection kit (KeyGEN, BioTECH, Nanjing, China) by flow cytometry, according to the manufacturer's instructions. FlowJo software was used for the data analysis (Leonard Herzenberg, Stanford University, USA). Cells staining negative in the presence of Annexin-V and PI were defined as viable cells.

Cell cycle analysis

MG-63 cells were seeded in 6-well plates (1×10^6 cells/well) and incubated at 37°C for 12 h. The target cells were treated with the compound (0.5 μ M) for 24 h. After treatment, cells were collected and fixed with 75% ethanol at -20°C overnight. In the next step, cells were washed with PBS followed by centrifugation, and incubated with 5 μ L (10 mg/mL) RNase and 2.5 μ L (5 mg/mL) propidium iodide (Beyotime Institute of Biotechnology, Haimen, China) for 30 min. Flow cytometry analysis was performed using CellQuest software (BD Bioscience, USA) and FlowJo software was used for the data analysis (Leonard Herzenberg, Stanford University, USA).

Statistical analysis

All experiments were performed at least 3 times. Values are expressed as the mean \pm standard deviation. Significant differences among the groups were determined by one-way analysis of variance using Origin8.6 (OriginLab Corporation, Northampton, MA, USA).

Results

Drug-target search

While osteosarcoma has been referred to as an "orphan cancer" with no known driver oncogenes [21], it actually was reported to include many useful biomarkers. In order to detect the potential targets for new drugs, we first focused on the clinically known drugs for OS therapy and sorted out 28 monomers (small molecules and monoclonal antibody, as presented in Table 1), as well as seeking corresponding targets of these drugs by searching in the DrugBank database [17]. Secondly, it is well known that there are hundreds of bioinformatic databases on various areas of molecular biology released in the past 10 years [22], forming the basis of "big data". Among them, Target Central Resource Database (TCRD, <https://pharos.nih.gov/idg/index>) is mainly curated interrelated data on unstudied and understudied drug targets. We collected 31 potential OS protein kinase targets (Table 2) by searching the TCRD database, taking into consideration their vital function of signaling pathways. Thirdly, a great many powerful studies indicated that miRNAs can effectively regulate OS progression [23–25], leading to the assumption that they should possess 1 or more common drug targets. Thus, searched for possible OS targets of miRNAs related to OS by querying high degree of confidence (Pred.Score ≥ 0.9) targets in Tarbase [14]. We found 171 OS targets.

Network construction and analysis

The 171 potential OS targets were imported into Database for Annotation, Visualization, and Integrated Discovery (DAVID) for mapping these targets into the KEGG pathway database.

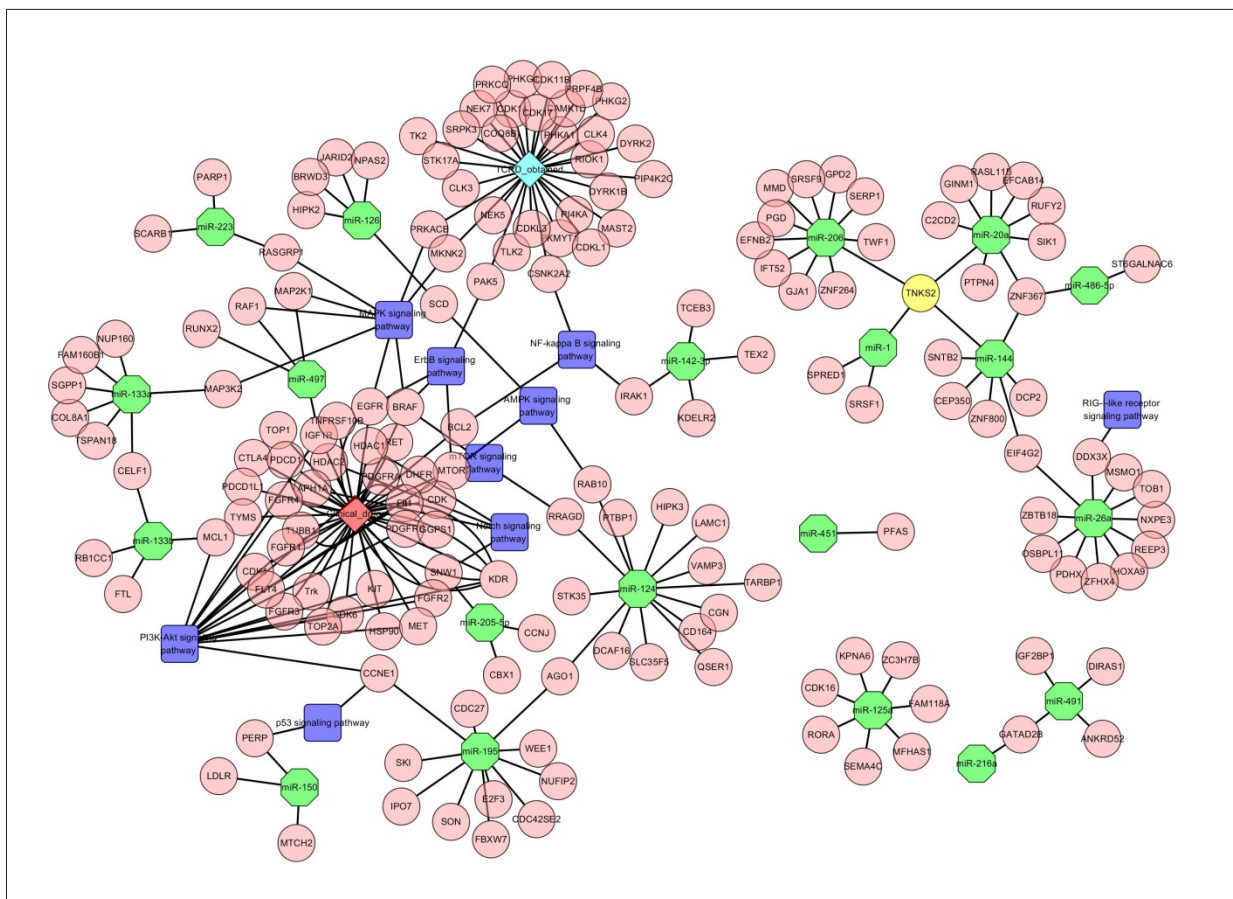


Figure 1. Network of “Target-Pathway” in osteosarcoma (OS) based on the collected data from Tables 1–4. Red rhombus: Clinical drugs for the treatment of OS; Cyan rhombus: TCRD database; Pink circle: potential OS targets; Green polygon: miRNA involved with OS; Blue rectangle: signaling pathway; Yellow circle: tankyrase 2 (TNSK2).

As shown in Figure 1, only a few key cell signaling pathways were manually retained, while there were many mapping items presented in the output of the DAVID website. The obtained results showed that the primary molecular mechanism of clinical drugs in Table 1 were the MAPK signaling pathway and the PI3K-Akt signaling pathway. Most of the targets validated from TCRD are shown in Table 2, but there were still several apparent protein targets such as PRKACB, MKNK2, and PAK5 that are closely involved with the MAPK signaling pathway and the ErbB signaling pathway. In addition, it is obvious that half of these miRNAs can also be mapped into these signaling pathways. Searching for new targets around these classical signaling pathways takes considerable effort, but selecting novel targets seemed more meaningful for the development of anti-OS drugs. A series of studies showing that miRNAs can perturb OS could reveal more potential targets. The localized network at the right part of Figure 1 shows that the 3 targets – TNSK2, ZNF367, and BIF4G2 – are also new possible targets in the further target validation stage.

TNSK2 inhibitor decreases OS cell proliferation

The present study evaluated the anti-proliferative activity effects of TNSK2 inhibitor on 4 OS cells (LM7, SaOS-2, U2OS, and MG-63) using the MTT assay. As shown in Figure 2, the target compound effectively inhibited the proliferation of all the tested OS cells at the concentration of 0.5 μM, and also exhibited more potent inhibition over time. Among them, the inhibitory activity against MG63 cells was most potent.

TNSK2 inhibitor induced G2/M phase arrest, and induced OS cell apoptosis in a dose-dependent manner

The TNSK2 inhibitor obviously induced G2/M phase arrest of MG63 cells at the concentration of 100 nM (Figure 3). It was apparent that the percentage of MG63 cells in the G2/M phase was also markedly increased (62% cells) when compared with the blank control. Figure 4 shows that TNSK2 inhibitor induced MG63 cell apoptosis in a dose-dependent manner. The percentage of apoptotic cells determined by flow cytometry analysis was obviously increased with increasing dose (10, 50, 100,

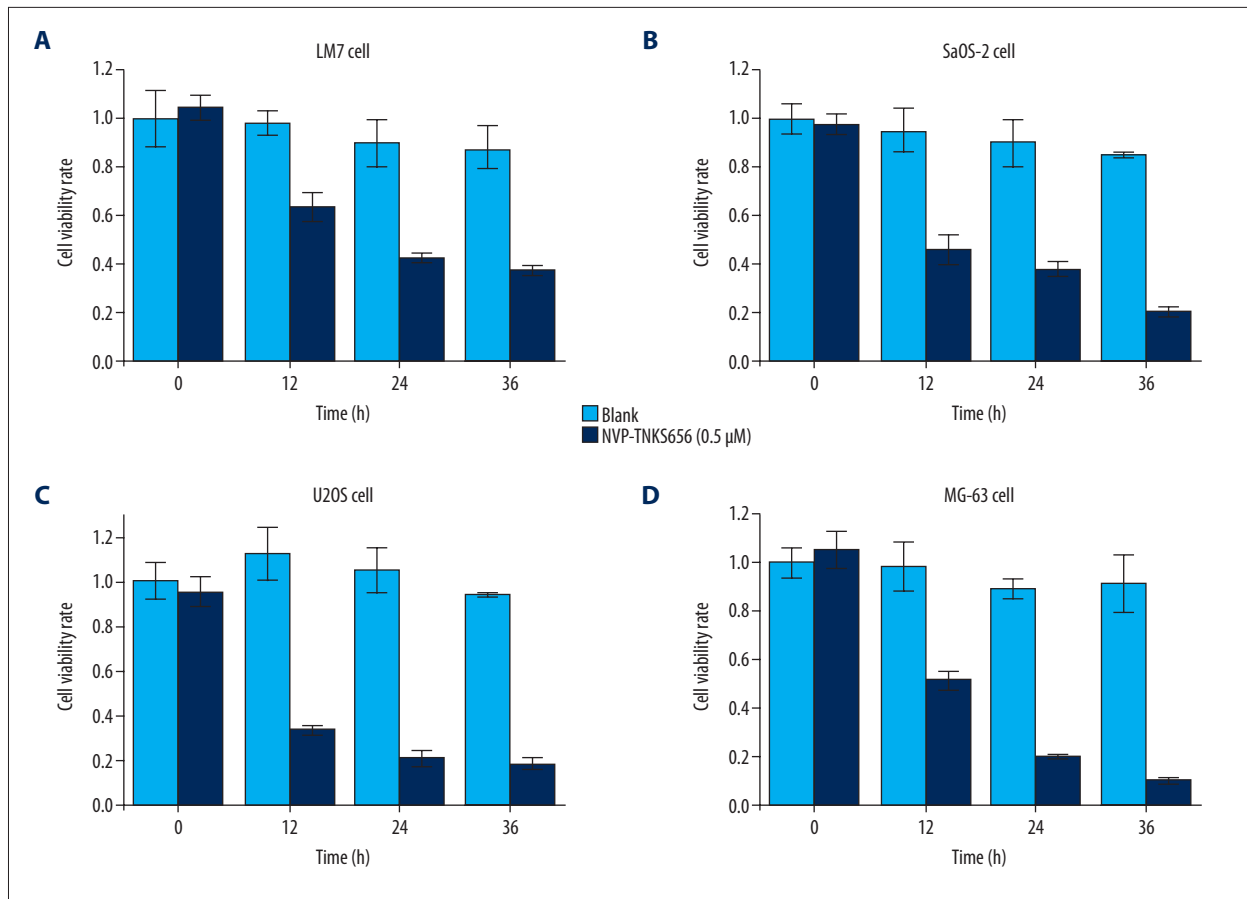


Figure 2. Antiproliferative activity of NVP-TNKS656 against the 4 common osteosarcoma cells at the increase of incubating time: 0, 12, 24, and 36 h after dosing. (A) LM7 cell; (B) SaOS-2 cell; (C) U2OS cell; (D) MG-63 cell.

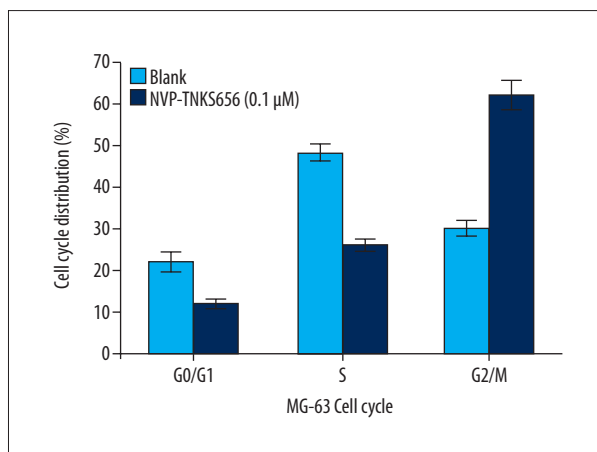


Figure 3. Comparison of the cell cycle distribution after drug treatment determined by flow cytometry using MG-63 osteosarcoma cells. Light blue bar: blank control; deep blue bar: NVP-TNKS656 at the concentration of 0.1 μM.

and 200 nM) of test compound. The apoptosis rate of MG63 osteosarcoma cells was 2.73%, 3.87%, 5.89%, and 6.97%, respectively. Moreover, the early apoptosis rate of MG63 cells

was 12.9%, even at the low concentration of NVP-TNKS656 (10 nM, Figure 4A).

Discussion

All the potential targets are presented in detail in Tables 1–4. In order to build the network more conveniently, the 28 drugs in Table 1 were named as “clinical drugs”. For the TCRD targets in Table 2, we attempted to connect them with the supposition “TCRD_Obtained”. The dataset in Tables 3 and 4 could be employed for the establishment of the “miRNA-Targets” network (Figure 1). As mentioned previously, the network of the signaling pathway is a great source of protein targets in OS cells, as well as other common cancer cells such as EGFR [26] located in the ErbB signaling pathway as a tumor marker or valid target used for the treatment of non-small cell lung cancer (NSCLC).

TNSKS2, which is a poly (ADP-ribose) polymerase (PARP) that adds ADP-ribose polymers onto target proteins [20]. In fact, as blockbuster PARP inhibitors such as Olaparib [27], Rucaparib [28], and Niraparib [29] have been approved as novel

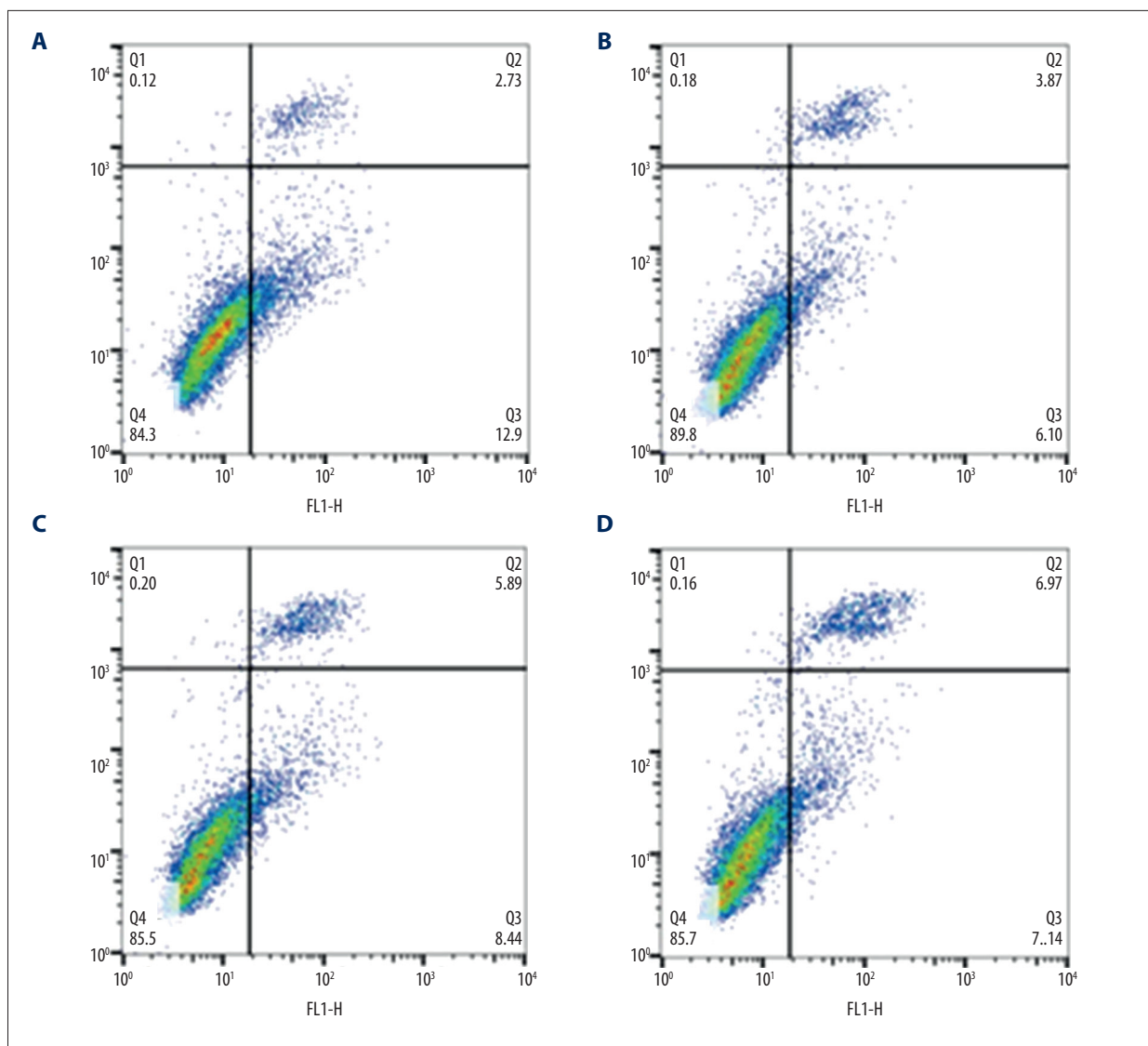


Figure 4. MG-63 cells treated with different concentrations of compound NVP-TNKS656 for 48 h were collected and cell apoptosis was analyzed by flow cytometry. (A) 10 nM; (B) 50 nM; (C) 100 nM; (D) 200 nM.

anticancer agents in the last 2 years, the development of PARP inhibitors has been regarded as the new direction of anticancer drugs. Moreover, TNKS2 was closely correlated with the Wnt/ β -catenin pathway, and the inhibition of TNKS2 can overcome resistance to PI3K/AKT inhibitors in cancer therapy [30]. However, there are only a few TNKS2 inhibitors reported to be in the preclinical phase so far. Based on the information above, we surmise that TNKS2 or function-similar protein might be regarded as a class of novel targets used for the treatment of OS. In this next section, we demonstrate the effectiveness for the inhibition of TNKS2 by preliminary biological assay.

Compound NVP-TNKS656 (Figure 5) was reported to be a highly potent and selective tankyrase inhibitor with the IC_{50} value of 6 nM against Tankyrase 2 (TNKS2). Tankyrase 2 is regarded

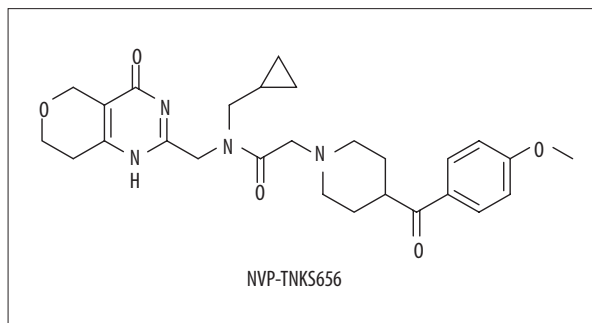


Figure 5. The chemical structure of NVP-TNKS656 as highly selective tankyrase 2 (TNKS2) inhibitor.

as a key druggable node in the Wnt pathway involved with carcinogenesis, and the 4-methoxybenzoyl-piperidine moiety of this compound is responsible for the inhibition of TNSK2. Actually, the idea of tankyrases as drug targets was put forward as early as 2013 [31], and most studies of tankyrase inhibitors [32-34] have proved them to be effective for specific cancers, possibly including OS. The MTT assay used in this study to a certain extent verified the potential of TNKS2 inhibitor, at least at the OS cell level.

In order to further investigate OS cell suppression by tankyrase inhibition, compound NVP-TNKS656 was chosen for further cell cycle and induced apoptosis assay. The G2/M checkpoint prevents cells from entering mitosis when unrepaired DNA damage was generated in cells [35]. Consistent with previous studies, several papers [30,36,37] on TNSK inhibitors reported that downregulation of the cell cycle regulator Cyclin D1 could explain the G2/M cell cycle, particular the "IWR-1" just published in the *Cancer Letters* journal [30]. Additionally, we also performed a cell apoptosis experiment to investigate whether the inhibitory activity of NVP-TNKS656 against MG63 cells was related to cell apoptosis. These 2 results provide further evidence that TNSK2 inhibitors have potential for the treatment of OS, and TNSK2 might be a promising target for the design of novel OS drugs.

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Conclusions

Osteosarcoma (OS) is one of the most common human cancers, but there is no clear and effective antitumor target, even for those known clinical candidates used for the treatment of OS, which is very challenging for the development of anti-osteosarcoma drugs. In the present study, we attempted to find more promising targets that might be used for OS therapy by construction and analysis of the "Target-Pathway" network. The obtained results show that tankyrase 2 (TNKS2), a multifunctional poly (ADP-ribose) polymerase (PARP), might be a very potential protein target for the treatment of osteosarcoma. An *in vitro* MTT assay proved that it is an available option against OS by targeting the TNKS2 protein. Subsequently, cell cycle and apoptosis assay by flow cytometry showed the TNKS2 inhibitor can obviously induce cell cycle arrest, apoptosis, and mitotic cell death. The present study shows a new direction directing for the development of anti-osteosarcoma drugs and provides a combined medication scheme for the treatment of OS.

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