In vitro, in *silico* and *in vivo* screening of non-oncology drugs for repurposing in osteosarcoma

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ABSTRACT: The first-line chemotherapy is associated with chief shortfalls such as non-specific distribution causing severe dose-dependent toxicities and development of tumor resistance. The current preliminary study aimed to identify the safe and effective non-oncology drugs as an alternative to toxic chemotherapeutics to treat osteosarcoma, and overcome new drug's shortage and development challenges. The different category non-oncology drugs (alone and in combinations) were screened for in vitro cytotoxicity behavior via MTT dye reduction assay and cell cycle arresting behavior using flow cytometer against human osteosarcoma (Saos-2 and MG-63) cells. The molecular docking of selected therapeutics was executed against cyclin-dependent kinase 1 (CDK1), cell cycle regulator overexpressed in cancer. The identified combination was further tested for in vivo toxicities in rats at two different doses. The current study revealed niclosamide (NSD), ketoconazole (KCZ), simvastatin (SVN) combination that causes substantial cytotoxicity (IC₅₀ values are in picomoles) at 1:1:3 molar ratio when compared to other molar ratios. This combination has also caused substantial arrest of Saos-2 and MG-63 cells at S and G2/M phase. Additionally, all three drugs demonstrated better interaction with CDK1 indicating anticancer potential via inhibition of CDK1. Furthermore, the in vivo toxicity study revealed no significant changes in hematological and biochemical parameters, body weights of rats, weights of vital organs, daily food and water intake, and general behavior of rats. The obtained preliminary results revealed the potential application of this combination on non-oncology drugs in the safe and effective treatment of osteosarcoma. However, further in-depth studies are required before clinical application.

KEYWORDS: Drug repurposing; osteosarcoma; cytotoxicity; molecular docking; acute toxicity.

1. INTRODUCTION

Cancer is one of the principal causes of mortality universally [1, 2]. The development of new pharmaceuticals with the goal of reducing mortality is fraught with difficulties [3, 4]; it takes an average of 13 years to translate new drugs into clinical practice; and the expected cost of new drug development will be between \$2 and \$3 billion USD. The practice of using medications that have been approved for one therapeutic application to treat a different ailment is known as drug repurposing [5, 6]. This approach is being applied more frequently to address the cancer drug shortage [7]. Moreover, this avenue proffers a new opportunity for the treatment of cancer, facilitating rapid clinical translation owing to the well known pharmacokinetic, pharmacodynamic, and toxicity profiles of these medications [8, 9]. Therefore, if new pharmaceuticals fail during research and development, this approach may lead to a less perilous business model with reduced development costs [10, 11]. Drug repurposing further increases the overall yield of

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drug discovery and rightfully concentrates on target-defined anti-neoplastic drugs with a better awareness of the ensign of cancer and the development of a range of data-driven methodologies. Additionally, it is important to take these non-oncology medications seriously because they may be able to target both known and previously unidentified cancer vulnerabilities. In fact, these old generic medications, which are typically used in a multi-target strategy and differ from targeted medications, may help patients [12].

Osteosarcoma is the most frequent primary pediatric bone cancer, developed from prehistoric boneforming (osteoid producing) mesenchymal cells. It constitutes of about 20% of all primary bone tumours and can be primary or secondary that has undergone malignant degeneration/conversion. Due to the significant heterogeneity of osteosarcoma, it can be categorized into an assorted types based on the degree of differentiation, locality within the bone, and histological disparity [13]. The most frequent primary bone tumour in children, teenagers, and young adults is osteosarcoma, which accounts for about 3.5% of all pediatric malignancies and 56% of pediatric malignant bone tumours. Its frequency rate varies amid 1 and 5 occurrences per million individuals, and patients between the ages of 10 and 19 are typically diagnosed with it [14].

Despite the fact that there is a dose effect on treatment response, numerous reports demonstrated that high-dose chemotherapy may not improve survival rates any more than less toxic moderate doses [15]. The anti-osteosarcoma drugs currently in use have a narrow therapeutic index due to the lack of tumour selectivity, metastatic events, or the multifarious etiology of these bone tumours16, and no improvement in survival rates has been made in the last three decades. In localized osteosarcoma, chemotherapy plus surgery result in a 5-year event-free survival rate of 60-70%, but little further development has been made in recent years [14]. Lack of new drugs in the pipeline makes it difficult to conduct clinical research on osteosarcoma. An intriguing solution to this problem is drug repurposing, an alternative development pathway that aims to use current medications as the foundation for fresh treatment possibilities with ability of targeting different cancer hallmarks.

Various non-oncology drugs including metformin (MFN), tadalafil (TDL), ketoconazole (KCZ), simvastatin (SVN), verapamil (VPL), and disulfiram (DSR), etc. have displayed potential anticancer activities against sorts of cancers including lung, liver, breast and melanoma [15-20]. Therefore, in the present research efforts have been made to preliminarily ascertain the in vitro and in silico anticancer activities of various non-oncology drug categories against human osteosarcoma cells, both singly and in combination. The goal of this preliminary study is to identify the combination of non-oncology drugs that has the most cytotoxic effect at the lowest dosage against osteosarcoma. Moreover, this combination will be tested in an animal model for any potential harm at varying concentrations.

2. RESULTS AND DISCUSSION

2.1. In vitro cytotoxicity study

The *in vitro* cytotoxicity of 11 drugs from different classes against human osteosarcoma cells (Saos-2 cells) is determined using MTT dye reduction assay. All drugs showed dose dependent Saos-2 cell growth inhibition (Figure 1A). NSD, MDZ, KCZ, and SVN showed remarkable cytotoxicity (lower IC₅₀ values) when compared to other drugs tested (Table 1). The IC₅₀ values of ALS, AMT and DSR are not calculated due to their lesser cytotoxic effect (<50% inhibition of Saos-2 cell growth).

As a result of the higher cytotoxicity NSD, KCZ and SVN, they were further screened in combinations at different molar ratios for their cytotoxicity behaviour against Saos-2 cells. The combinations at different molar ratios showed dose dependent Saos-2 cell growth inhibition (Figure 1B). The IC₅₀ values obtained for combinations of different molar ratios are depicted in Table 1. The NSD+KCZ+SVN combination at 1:1:3 ratio exhibited substantially higher cytotoxicity (lower IC₅₀ value) when compared to other molar ratios after 48h of treatment. Furthermore, the anticancer potential of NSD+KCZ+SVN combination at 1:1:3 is also validated against MG-63 cells (other human osteosarcoma cells). This combination displayed concentration dependent cytotoxicity against MG-63 cells after 48h of treatment (Figure 2C). The IC₅₀ value calculated is 0.453±0.051nM for MG-63 cells after 48h of treatment. Besides, the cytotoxic nature of NSD+KCZ+SVN combination (at 1:1:3 molar ration) is also tested against human healthy kidney cells (HEK-293). This combination caused substantially lowest cell growth inhibition (only 27.98±3.5%) after 48h treatment (Figure 1C). The above results clearly indicate the repurposing potential of NSD+KCZ+SVN combination in osteosarcoma treatment.



Figure 1 (A) & (B): % Saos-2 cell growth inhibition caused by different test solutions after 48h of incubation; **(C):** Effect of NSD, KCZ, and SVN combination at 1:1:3 molar ratio on MG-63 and HEK-293 cell growth inhibition. The X-axis concentration values are negative because during the analysis the software converts the linear values into log values.

Table 1 The IC₅₀ values of different drugs and their combinations after 48 h treatment of Saos-2 cells

Saos-2 cell	s	Saos-2 c	ells	
Samala Nama	IC ₅₀ Values (µM)	Samula Nama	IC ₅₀ Values (nM)	
Sample Name	(10-0.0001µM)	Sample Name	(100-0.001nM)	
Alendronate Sodium (ALS)	ND	NSD+KCZ+SVN (1:1:1)	0.181±0.021	
Dexamethasone (DMS)	1.854 ± 0.34	NSD+KCZ+SVN (1:1:2)	0.119±0.023	
Niclosamide (NSD)	0.148 ± 0.06	NSD+KCZ+SVN (1:1:3)	0.088 ± 0.006	
Mebendazole (MDZ)	0.131±0.01	NSD+KCZ+SVN (1:2:1)	0.232±0.023	
Tadalafil (TDL)	1.288 ± 0.18	NSD+KCZ+SVN (1:3:1)	0.298±0.022	
Metformin (MFN)	1.089±0.11	NSD+KCZ+SVN (2:1:1)	0.6595 ± 0.123	
Ketoconazole (KCZ)	0.126±0.04	NSD+KCZ+SVN (3:1:1)	0.9535±0.212	
Simvastatin (SVN)	0.103±0.06			
Aprimilast (AMT)	ND			
Verapamil (VPL)	1.193±0.34			
Disulfiram (DSR)	ND			

Values presented are Mean±SD, ND: Not detected due to lesser cell inhibition

2.2. Cell cycle analysis by flow cytometer

In the present study, the effect of NSD, MDZ, KCZ and SVN on Saos-2 cell replication stages is determined (Table 2). A marked reduction in G0/G1 phase was observed after treatment with NSD, MDZ, KCZ and SVN when compared to untreated control cells. A remarkable accumulation of Saos-2 cells at S-phase was noticed with NSD, MDZ, KCZ and SVN treatments when compared to untreated cells. Furthermore, moderate to substantial Saos-2 cell arrest at G2/M phase is observed with MDZ and KCZ treatment, respectively. In addition, the NSD+KCZ+SVN (100pM:100pM:300pM) combination at lowest concentration caused substantially increased SaoS-2 cells arrest at S and G2/M phase. All three drugs displayed cell cycle arrest at S-phase (Figure 2). This S-phase (synthesis phase) of the cell cycle is related to the synthesis and replication of DNA. This combination effect is further confirmed using other human osteosarcoma cells (MG-63 cells). The obtained results indicate superior cell cycle arresting behavior of

NSD+KCZ+SVN combination at lowest concentration; thus, this combination could be potentially used in osteosarcoma treatment.

 Table 2 Effect of test formulations on different cell cycle phases of different human osteosarcoma cells after 48h treatment

Cell line	Test sample	Concentration	SUBG0	G0/G1	S	G2M
SaOS-2	Control		0.59±0.06	90.41±5.6	2.41±0.71	13.26±1.33
	NSD	0.2µM	3.83±0.12	69.98±4.1	16.79±0.95	10.67±1.33
	MDZ	0.2µM	0.74 ± 0.05	66.33±3.5	17.01±1.1	16.06±0.8
	KCZ	0.2µM	0.74 ± 0.04	60.87±2.7	9.8±0.3	29.19±1.9
	SVN	0.2µM	0.06 ± 0.01	71.34±3.9	14.39±0.7	14.75±0.8
SaOS-2	Control		2.41±0.5	55.88±6.6	6.66±0.9	5.37 ± 0.4
	NSD:KCZ:SVN (1:1:3 molar ratio)	100pM:100pM:300pM	0.83±0.04	59.88±3.1	12.41±0.82	27.43±2.12
MG-63	Control		3.57 ± 0.7	88.69±4.8	3.36±0.5	4.95 ± 0.4
	NSD:KCZ:SVN (1:1:3 molar ratio)	100pM:100pM:300pM	0.84±0.05	69.98±4.6	10.63±1.1	19.07±1.4

Values presented are Mean±SD, n=3.



Figure 2: Cell cycle phases of Saos-2 and MG-63 cells after 48h treatment with different test solutions.

2.3. Molecular docking

The cell cycle kinases enzyme like cyclin-dependent kinase 1 (CDK1) are serine or threonine kinases whose catalytic activities are controlled *via* interactions with cyclins and CDK inhibitors. CDK1 assume an imperative role in the cell cycle progression *via* regulating cell-cycle checkpoints and transcriptional events in response to extracellular and intracellular signals. Phosphorylation of CDKs occurs following complex formation with cyclins. Different CDKs (CDK1, CDK2, CDK3, CDK4, CDK6, and CDK7) are in the cell division and regulation [21]. Various studies reported upregulated level of CDK1 in tumors including osteosarcoma. Thus, the upregulated level of CDK1 causes augments in cell proliferation [22-24]. The CDK1, therefore, serves as a target of therapeutics of cancer treatment [21-25]. The sorts of CDK1 inhibitors found to be promising in the treatment of variety of cancer including breast cancer and osteosarcoma.

CDK1/Cks2 (cyclin dependent kinase subunit 2) is expressed in osteosarcoma. This CDK1 protein was reported to be involved in the progression and regulation of the cell cycle where its function is synchronized by binding with Cks subunit [26]. Thus, targeting CDK1 protein can be a promising approach

in the treatment of osteosarcoma. *In silico* techniques can assist to recognize the potential of repurposed therapeutics against tumor cells having upregulated proteins like CDK1. Therefore, in the present preliminary research, KCZ, NSD and SVN are docked with target protein CDK1/Cks2. Molecular docking was validated using redocking of dinaciclib with CDK1/Cks2, the RMSD of the re-docking was observed to be 1.228 angstrom. Co-crystallized reference ligand from PDB ID: 6GU6 i.e. Dinaciclib was re-docked with the CDK1/Cks2 and obtained binding affinity and interactions were compared with KCZ, NSD, and SVN, respectively. Dinaciclib showed a binding affinity of -9.0 kcal/mol. Dinaciclib formed 5-hydrogen bonds (3 conventional and 2 carbon-hydrogen bonds) with ILE10, LEU83, ASP146, GLU81, and ASN133. Hydrophobic interactions (alkyl and Pi-alkyl) formed between VAL64, ALA31, ALA145, and VAL18. Pi-sigma interactions were formed between LEU135 and PHE80 (Figure 3A & 3B).

Antifungal KCZ displayed a stable complex with CDK1/Cks2 *via* -7 kcal/mol binding energy. KCZ formed 2-hydrogen bonds (1-conventional and 1-carbon-hydrogen) with GLY154 and ARG36. Moreover, many hydrophobic interactions (Pi-alkyl) with ILE35 and ILE155 and van der Waals interactions with GLU38, VAL44, PHE153, VAL44, GLU41, GLU41, GLU40, SER39, SER46, TYR15, LEU37 were also observed (Figure 3C & 3D). Similarly, NSD (well known anthelmintic agent) was found to be forming a stable complex with CDK1/Cks2 *via* -6.4 kcal/mol binding energy. It formed 2-hydrogen bonds (1-conventional and 1-Pi-Donor hydrogen) with LYS33, PHE82, and hydrophobic interaction (Pi-alkyl) with VAL18, Pi-cation with LYS89, Pi-Sigma with ILE10 and van der Waals interaction with GLY11, ASP86, LEU135, ALA145, ASP146, ASN133, THR14, GLY13, LEU83, GLN132, SER84 (Figure 3E & 3F).

Furthermore, antihyperlipidemic SVN was also found to be forming a stable complex with CDK1/Cks2 *via* -5.8 kcal/mol binding energy. SVN formed 5-hydrogen bonds (4-conventional and 1-carbonhydrogen) with ALA48, GLU38, ARG36, GLY154, and SER46 and hydrophobic interaction (alkyl) with LEU37 and van der Waals interaction with THR47, ILE35, TYR15, PHE153, ILE155, ALA152 (Figure 3G & 3H). The above docking study results obtained for NSD, KCZ and SVN are supporting the cell cycle arresting characteristics determined using flow cytometer.



Figure 3 (A) & (B): 2D and 3D images of interaction of ketoconazole with CDK1/Cks2; (C) & (D): 2D and 3D images of interaction of niclosamide with CDK1/Cks2, and (E) & (F): 2D and 3D images of interaction of simvastatin with CDK1/Cks2

2.4. In vivo toxicity study

The *in vivo* toxicity of NSD+KCZ+SVN combination of 1:1:3 molar ratio at two different doses is determined following intravenous administration at every 2nd day up to 14 days in rats. At the end of the study (on 14th day), the consequences of test solutions on hematological (Table 3) and biochemical (serum) parameters (Table 4) are determined. There are no substantial changes in the hematological and biochemical parameters of drug treated groups (Group C & D) when compared to the untreated control group (Group A). These results indicate better safety of the test formulation at both doses. However, quite altered few hematological and serum parameters with the high dose of the test solution in Group D are noticed.

Additionally, the animals are measured for body weights during the study and as well as the weights of their vital organs (Table 5). After all, the moderately augmented body weight is seen in the untreated control group, vehicle control, and group C treated with lower (dose 1) test formulation. However, about 2%

decreased body weight is observed with the group D treated with higher dose (0.3mL). Besides, there are no substantial changes in the organ's weights, food intake, and water intake is observed in comparison to untreated control group (Table 4). Furthermore, no death of animal is observed from group C and D treated with formulations. These obtained results further indicate the safety of test formulation at both the doses.

Table 3: Summary of haematological parameters observed at the end of the study						
Haematological Parameters	Group-A	Group-B	Group-C	Group-D		
WBC (10^3/cumm)	9.6±2.82	11.86±2.3	14.66±8.98	9.26±1.16		
RBC (10^3/cumm)	7.47±0.38	8.66±0.77	7.8±0.37	8.65±1.36		
Hb (gm%)	12.85±0.77	14.2±0.72	12.96±0.32	14.13±1.0		
Neutrophils (%)	63±4.24	58.33±4.93	63.33±15.17	48±4.35		
Lymphocytes (%)	33.5±2.12	35.66±4.04	30±16	45.33±3.78		
Monocytes (%)	1±0.01	2.33±2.3	3±0.02	3±0.01		
Eosinophils (%)	2.5±2.12	3.66±1.15	3.66±2.51	3.33±0.57		
PCV (%)	39.9±2.4	43.53±2.67	39.86±0.75	43.4±4		
M.C.V (fl)	54.05±0.49	50.3±1.51	51.16±2.08	50.5±3.41		
M.C.H (pg)	17.4±0.14	16.43±0.68	16.66±0.81	16.5±1.53		
M.C.H.C (g/dl)	32.2±0.13	32.63±0.41	32.5±0.3	32.6±0.86		

Values presented are mean ± SD. **Group - A:** Untreated control. **Group - B:** Vehicle control (0.3mL). **Group -C:** Dose 1 (3:1:1 molar ratio; 0.1mL) (SVN: 0.153mg; KCZ: 0.06469mg; NSD: 0.03983mg). **Group - D:** Dose 2 (Higher dose of 3:1:1 molar ratio; 0.3mL) (SVN: 0.459mg; KCZ: 0.194mg; NSD: 0.1194mg).

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Biochemical Parameters	Group-A	Group-B	Group-C	Group-D
Total bilirubin (mg/dl)	0.25 ± 0.09	0.25±0.09	0.25±0.09	0.25±0.09
Direct bilirubin (mg/dl)	0.15 ± 0.05	0.15 ± 0.05	0.15 ± 0.05	0.15 ± 0.05
Indirect bilirubin (mg/dl)	0.13 ± 0.14	0.13 ± 0.14	0.13 ± 0.14	0.13 ± 0.14
Total protein (g/dl)	5.69±1.26	5.69±1.26	5.69±1.26	5.69±1.26
Serum albumin (g/dl)	4.69 ± 0.71	4.69 ± 0.71	4.69 ± 0.71	4.69±0.71
Serum globulin (g/dl)	1±0.55	1±0.55	1±0.55	1±0.55
A/G ratio	5.3±2.21	5.3±2.21	5.3±2.21	5.3±2.21
SGOT (U/L)	14.87 ± 3.48	14.87 ± 3.48	14.87 ± 3.48	14.87 ± 3.48
SGPT (U/L)	70.09 ± 2.24	70.09 ± 2.24	70.09 ± 2.24	70.09±2.24
ALP (U/L)	456.8±189.25	456.8±189.25	456.8±189.25	456.8±189.25
Urea (mg/dl)	29.73±4.72	29.73±4.72	29.73±4.72	29.73±4.72
Creatinine (mg/dl)	0.28±0.19	0.28±0.19	0.28±0.19	0.28±0.19

Values presented are mean ± SD. **Group - A:** Untreated control. **Group - B:** Vehicle control (0.3mL). **Group - C:** Dose 1 (3:1:1 molar ratio; 0.1mL) (SVN: 0.153mg; KCZ: 0.06469mg; NSD: 0.03983mg). **Group - D:** Dose 2 (Higher dose of 3:1:1 molar ratio; 0.3mL) (SVN: 0.459mg; KCZ: 0.194mg; NSD: 0.1194mg).

Table 5: %	average group boo	ly weight and	weights of v	ital organs m	easured during	g and after	the study
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	Group-A	Group-B	Group-C	Group-D				
Mean body weight inclined (+)/ declined (-) (%):								
	+5.3	+3.8	+2.3	-2.0				
Organ weight (g):								
Liver	9.21±0.12	9.12±0.23	9.09±0.09	8.89±0.12				
Heart	1.24±0.05	1.29±0.04	1.21 ± 0.01	1.24±0.03				
Lungs	1.98±0.13	1.93±0.25	2.10±0.34	1.90±0.23				
Spleen	0.64±0.03	0.65 ± 0.04	0.69±0.02	0.67 ± 0.01				
Brain	1.53±0.05	1.57±0.03	1.55 ± 0.01	1.45 ± 0.02				
Kidney (Right)	1.16±0.05	1.14±0.03	1.14 ± 0.01	1.10±0.02				
Kidney (Left)	1.13±0.02	1.09 ± 0.04	1.11±0.02	1.10 ± 0.01				

Values presented are mean ± SD. **Group - A:** Untreated control. **Group - B:** Vehicle control (0.3mL). **Group -C:** Dose 1 (3:1:1 molar ratio; 0.1mL) (SVN: 0.153mg; KCZ: 0.06469mg; NSD: 0.03983mg). **Group - D:** Dose 2 (Higher dose of 3:1:1 molar ratio; 0.3mL) (SVN: 0.459mg; KCZ: 0.194mg; NSD: 0.1194mg).

3. CONCLUSION

The present preliminary investigation has identified a combination of non-oncology drugs (Niclosamide Ketoconazole, and Simvastatin) for the treatment of osteosarcoma. The substantial *in vitro* anticancer effect of this combination at the lowest concentration against human osteosarcoma cells indicates its potential clinical applications in the treatment of osteosarcoma. Moreover, an *in silico* study showed strong interactions with all the docked ligand groups; hence the formed complex has stable binding. Thus, the obtained results suggest the possibility of inhibiting CDK1 protein (cell cycle regulator) by all three therapeutics; however, additional molecular mechanism-based studies are needed to validate these results. Besides, further studies are needed to decide the right route for administration, the right composition, and right dosage form for effective treatment of osteosarcoma.

4. MATERIALS AND METHODS

4.1. Materials

Niclosamide (NSD), ketoconazole (KCZ), simvastatin (SVN), and propidium iodide were procured from Sigma Aldrich, Mumbai. MTT Powder, fetal bovine serum (FBS), PenStrep, trypsin, and Dulbecco's Modified Eagle Medium (DMEM) were procured from Invitrogen, Bangalore. RNase A was procured from Bochringer Mannhein GmbH, Germany.

4.2. Cell Culture

Saos-2, MG-63, and HEK-293 cells were procured from ATCC, USA. Saos-2 cells were cultured in RPMI medium, and MG-63 and HEK-293 cells were cultured in DMEM medium. The cells were developed in humidified atmosphere (5% CO₂ at 37°C) employing a media containing inactivated FBS (10%), penicillin (100 IU/mL), streptomycin (100 μ g/mL). The cells were dissociating solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS) were employed to dissociate cells.

4.3. Screening of anticancer efficacy of non-oncology drugs against hepatic cancer cells

4.3.1. In vitro cytotoxicity

In vitro cytotoxicity of repurposed drugs alone was initially tested against human osteosarcoma cells (Saos-2 cells). Briefly, cells were added to a 96-well plate and overnight incubated at 37°C. The cells were then treated with serially diluted test solutions (100 μ L each) and plates were incubated for another 48h. Then 100 μ L of MTT (6 mg/10mL of MTT in PBS) was added in the plates by removing test solutions and plates were further incubated for 4h in an analogous environment. Finally, the buoyant was discarded and

formazan crystals produced in viable cells were solubilized by DMSO (100μ L). The absorbance of the consequent solution was measured at 570 nm by a microplate. The IC₅₀ values were then calculated using dose-response curves [27].

By using the above procedure, the drugs exhibiting higher cytotoxicity (low IC_{50} values) were then further screened in combinations against Saos-2 cells. Furthermore, the cytotoxicity of the selected combination was further validated using other osteosarcoma cell (MG-63 cells), and the toxicity of combination to healthy cells (human embryonic kidney cells, HEK-293) was also studied.

4.3.2. Cell cycle analysis by flowcytometer

The cell cycle arresting behavior of NSD, KCZ, and SVN (selected based on their higher *in vitro* cytotoxicity behaviour) was determined using Saos-2 cells. To the 6-well plate with medium (2mL), 1x10⁶ cells were seeded and cultured for 24h. Following 48h of incubation, cells were treated with NSD, KCZ and SVN solutions of specific concentration, harvested and centrifuged at 2000 rpm for 5 min. The obtained cell pellet was repeatedly washed by using a 1X PBS (2mL). Cells were resuspended in a sheath fluid (300µL) and fixed by addition of chilled 70% EtOH (1mL) and mild shaking, and stored overnight at 4°C. Then cells were again centrifuged at same conditions as described above and washed for two times *via* cold 1X PBS (2mL) and resuspended in sheath fluid (450µL) containing 0.05mg/mL each of propidium iodide (PI) and RNase A, and incubated for 15 min in dark. The % of cells in an assortment of cell cycle stages in treated and untreated populations were determined *via* FACS Caliber (BD Biosciences, San Jose, CA) [28, 29]. Furthermore, the combination effect of NSD, KCZ, and SVN at 1:1:3 molar ratio on Saos-2 and MG-63 cells cycle stages was also determined using the above-described procedure.

4.4. Molecular docking study

Molecular docking was performed using PyRx-virtual screening tool [30]. The structures of all the drugs (NSD, KCZ and SVN) were downloaded in .sdf File format from National Center for Biotechnology Information PubChem database (https://pubchem.ncbi.nlm.nih.gov/). These drugs were docked against CDK1/Cks2 protein. The energy minimization (optimization) was performed by Universal Force Field (UFF). The structure of CDK1/Cks2 in complex with Dinaciclib (PDB ID: 6GU6, https://www.rcsb.org/structure/6gu6) was obtained from Protein Data Bank (PDB) www.rcsb.org. Autodock vina 1.1.2 in PyRx 0.8 was used to perform the MD studies of all the selected drugs against the crystal structure of CDK1/Cks2. The grid box was selected to cover the residues of the binding site, with center X: 22.3713, Y: 11.2505, Z: 12.1321, and with dimensions X: 56.5140, Y: 64.406828918, Z: 67.1576536751. Docked binding mode of ligands with highest negative binding affinity was selected and saved as a PDB file and 2D and 3D interactions was visualized by using BIOVIA Discovery Studio Visualizer [31].

4.5. In vivo toxicity study

Albino rats of either sex ranging from 200 to 250g were provided by Animal Care Facility, Tatyasaheb Kore College of Pharmacy, Warananagar (Protocol No.: TKCP/2021/08/07). All in vivo experiments were approved by the Animal Care and Use Committee. All care and handling of animals were performed with the approval of the review board of animal experiments. The Albino rats were randomly assigned into 4 groups (4 rats per group): The group (A) is untreated control group. The group (B) received intravenously 0.3mL of blank vehicle. The group (C) received intravenously 0.1mL of vehicle containing SVN (0.153mg; 0.0003655mM), KCZ (0.06469mg; 0.0001218mM), and NSD (0.03983mg; 0.0001218mM) at 3:1:1 mM ratio. The test solution (10mL) containing SVN (15.3mg), KCZ (6.469mg), and NSD (3.983mg) was prepared as discussed below. The vehicle used to dissolve above drugs is a mixture of 0.5mL of sterile DMSO, 0.5mL of sterile Tween 80 and 0.5mL of ethanol. To this vehicle mixture the SVN (15.3mg) was added first and dissolved using bath sonicator followed by KCZ (6.469mg) and NSD (3.983mg) were added and dissolved using bath sonicator. The obtained clear drug solution was further diluted to 10mL with sterile water for injection in a biosafety cabinet. In order to test toxicities of the above composition at higher dose the group (D) was administered with higher dose (0.3 mL) of the above solution. The 0.3 mL of the above test solution is composed of 0.459mg of SVN, 0.194mg of KCZ, and 0.1194mg of NSD. The above doses administered fall within the human therapeutic dose range.

The doses were administered to all group rats at every 2nd day *via* tail vein up to 14 days. Prior to the treatment, at every 2nd day during the treatment, and at the end of the experiment, the body weights, food consumption and water intake of animals were recorded. Additionally, the rats were noticed for the general toxicity signs such as impact on locomotion, behavior (agitation, decreased activity, and somnolence), etc

throughout treatment. On 14th day, the blood samples were withdrawn and subjected for hematological and biochemical evaluations. The above outcomes were employed as indicators of systemic toxicity of formulations [27, 32].

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