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Antitumour, acute toxicity and molecular modeling studies of 4-(pyridin-4-yl)-6-(thiophen-2-yl) pyrimidin-2(1H)-one against Ehrlich ascites carcinoma and sarcoma-180

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Abstract

In an effort to discover an effective and selective antitumour agent, synthesis and anti-cancer potential of 4-(pyridin-4-yl)-6-(thiophen-2-yl) pyrimidin-2(1*H*)-one (**SK-25**), which has been reported earlier by us with significant cytotoxicity towards MiaPaCa-2 malignant cells, with an IC_{50} value of 1.95 μM and was found to

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instigate apoptosis. In the present study, the antitumour efficacy of **SK-25** was investigated on Ehrlich ascites tumour (EAT, solid), Sarcoma 180 (solid) tumour and Ehrlich ascites carcinoma. The compound was found to inhibit tumour development by 94.71% in Ehrlich ascites carcinoma (EAC), 59.06% in Ehrlich tumour (ET, solid) and 45.68% in Sarcoma-180 (solid) at 30 mg/kg dose. Additionally, **SK-25** was established to be non-toxic at a maximum tolerated dose of 1000 mg/kg in acute oral toxicity in Swiss-albino mice. Computer-based predictions also show that the compounds could have an interesting DMPK profile since all 51 computed physicochemical parameters fall within the recommended range for 95% of known drugs. The current study provides insight for further investigation of the antitumour potential of the molecule.

Keywords: Pharmaceutical science, Cancer research, Pharmaceutical chemistry

1. Introduction

Cancer is the main cause of human deaths in economically advanced countries, having devastating effects. This is irrespective of the significant advancement in therapeutic innovation towards its diagnosis and treatment [1]. Although patients in these countries have access to state of the art chemotherapeutics and major steps taken towards the clinical management of the disease, the dilemma of undesirable side effects and emergent resistance of malignant cells to drugs has made numerous regimens ineffective. Thus, major efforts must be dedicated to hunting for innovative therapeutic candidates [2].

New anticancer drug candidates are often either designed on the basis of designing small molecules against specific targets or tumour types or by large-scale drug screening methods, viz; virtual screening of electronic libraries of high throughput screening of compound collections. Due to the specificity of different cancer types, such drug candidates must show higher efficacy and lesser side effects than known cytotoxic agents. The investigation of new antitumour agents often couples *in silico*, *in vitro* and *in vivo* techniques. *In vitro* screening is one of the prime fundamental steps (sometimes preceded by the *in silico* screening of millions of virtual compounds), aimed at identifying preliminary hits for further drug development. *In silico* and *in vitro* techniques are relatively less costly and less tedious, thus permitting the evaluation of more drug candidates, when compared with *in vivo* methods. For the aforementioned reasons, it is practically impossible to generate *in vivo* data for large datasets of anticancer drug candidates. However, *in vitro* experiments mainly serve to select the preliminary lead compound(s), which could then be further investigated in living organisms (*in vivo*) [3, 4]. A step-wise process is often followed from *in vitro* to *in vivo* to reduce the number of potential anticancer agents to be tested to just a few candidates, which could then be further taken to clinical trials. The current study reports *in vivo* studies of one of our previously reported potent anticancer chalcones

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on mice. This rodent was selected because their genetic, biological and behavioral characteristics closely related with humans [5].

Chalcones are a class of biaryl propenones, which have demonstrated toxicity against diverse malignant cells *via* the interaction with tubulin at its colchicine fastening position [6, 7, 8, 9, 10]. Chalcones, considered as the starting materials of flavonoids and isoflavonoids, have a broad range of pharmacological properties, including antitumour [11, 12, 13], anti-inflammatory [14, 15], anti-fungal and anti-tubercular properties [16]. Chemically, chalcones (1,3-diaryl-2- propen-1-ones) hold an enone connection among two aromatic rings [17, 18, 19, 20, 21, 22, 23]. Both naturally occurring and synthetic chalcones have revealed interesting biological profiles. They have served as lead compounds for the discovery of new anti-inflammatory, anti-infective, anti-cancer and antioxidant agents [24]. A literature report shows that chalcones are capable of inducing the death of cancer cells through apoptosis, as well as subsiding the mitochondrial membrane potential [25, 26, 27].

With the quest to discover safe and potent prospective novel chalcones as anticancer agents, a novel 4,6-diaryl pyrimidone derivative, *4-(pyridin-4-yl)-6-(thiophen-2-yl) pyrimidin-2(1H)-one* (**SK-25**) was reported by our research group, which involved the fusion of pyrimidone and chalcone to a rigid chalcone framework (Fig. 1) [28]. The aim of the current study is to investigate the *in vivo* anti-cancer potential of the synthesized compound, which restrains the tumour enlargement in tumour mice models like Ehrlich tumour (ET, solid), EAC and sarcoma-180 (solid). In addition, the acute oral toxicity of **SK-25** was also performed as per OECD guidelines

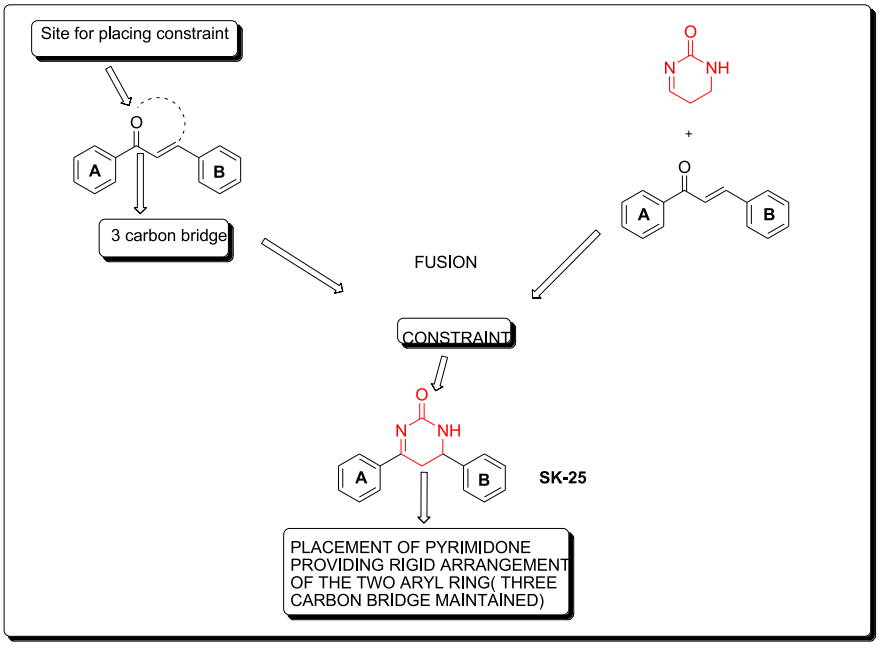

Fig. 1. Design strategy for the synthesis of SK-25.

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Table 1. Profile of optimized lead compound **SK-25**.

Code	**SK-25**			
Molecular formula	$C_{13}H_9N_3OS$			
IUPAC name	*4-(pyridin-4-yl)-6-(thiophen-2-yl) pyrimidin-2(1H)-one*			
State	Solid, powder			
Color	Light brown			
Structure	See Fig. 1			
Melting point	275-276 °C			
Yield	88 %			
^{1}H NMR	(DMSO-d_6, 300 MHz, δ, TMS = 0): 8.73 (2H, *d*, *J* = 4 Hz), 8.19 (1H, *d*, *J* = 4 Hz), 8.00 (2H, *bs*), 7.83 (2H, *m*), 7.23 (1H, *dd*, *J* = 2 and 4 Hz)			
^{13}C NMR	164.7, 163.4, 152.4, 149.9, 135.5, 134.3, 132.6, 129.7, 128.8, 128.4, 127.6, 124.3, 106.			
Percentage growth Inhibition at 50 μM	Pancreatic (MiaPaCa-2) 93	Prostate (PC-3) 13	Lung (A-549) –	Colon (HCT-116) 49
Elemental analysis	C, 61.16; H, 3.55; N, 16.46; S, 12.56 Found: C, 60.97; H, 3.66; N, 16.09; S, 12.44.			
IC_{50} Values	1.95 μM			
MMP Loss	51.2 % at 20 μM			
Apoptosis Induction	30.33 % at 20 μM			
Ehrlich ascitic carcinoma (EAC)	% Tumor cell growth		% Tumor growth inhibition	
20 mg/kg	8.43		91.56	

(*continued on next page*)

https://doi.org/10.1016/j.heliyon.2018.e00661

Table 1. (*Continued*)

Code	SK-25	
30 mg/kg	5.28	94.71
Ehrlich tumor (Solid)	Average tumor weight in mg	% Tumor growth inhibition
20 mg/kg	719.85 ± 62.35	38.64
30 mg/kg	480.28 ± 72.04	59.06
Sarcoma-180 (Solid)		
20 mg/kg	816.78 ± 120.89	32.90
30 mg/kg	661.14 ± 95	45.68
Toxicity	Acute oral-well tolerated up to 1000 mg/kg single dose	
Observational parameters	Normal	
Gross pathological Changes	No change	
Histopathological study	No change	

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Article No~e00661

No 423. This compound was observed to be most dynamic and potent with an IC_{50} value of 1.95 μM against MiaPaCa-2 cell lines and findings of the optimized lead compound are also summarized in tabular form in (Table 1).

2. Materials and methods

2.1. Chemicals

All the chemicals and reagents were purchased from Merck, CDH, Sigma Aldrich, Spectro chem., Loba chem., India and utilized without additional purification. Biotage Microwave Synthesizer (Model: Initiator) was used for carrying out the reactions operating at 150 °C, with the microwave power maximum level of 400 W. ^{1}H NMR and ^{13}C NMR (75 MHz) spectra were recorded using JEOL (300 MHz) NMR spectrometer. The spectra were determined in DMSO-d_6 relative to TMS (0.00 ppm). Chemical shifts were reported in δ values utilizing tetramethylsilane as an internal standard with the number of protons, multiplicities (s-singlet, d-doublet, t-triplet, q-quartet, m-multiplet, dd-double doublet) and coupling constants (J) in Hz (Hertz) in ^{1}H NMR. Melting points were determined in open capillaries and were uncorrected.

2.2. Chemistry

2.2.1. Preparation of tetrafluoroboric acid catalyst (HBF_4-SiO_2)

The tetrafluoroboric acid catalyst system was prepared following the formerly reported strategy [29]. About 8.25 ml of 40% aq. HBF_4 acid (3.3 g, 8.25 mL, 15 mmol) and 26.7 g of silica gel (300−400 mesh) were weighed and placed in round bottom flask containing 75 ml of diethyl ether and stirred for 3 hours on a magnetic stirrer. The above contents of the mixture were concentrated on rota evaporator and the deposits were dried under the vacuum for 72 h at 100 °C to get as a free-flowing powder of HBF_4-SiO_2 (0.5 mmol HBF_4/g) [30].

2.2.2. General method for the synthesis and characterization of 4-(pyridin-4-yl)-6-(thiophen-2-yl) pyrimidin-2(1H)-one (SK-25)

This was conducted as previously described [28, 30]. The compound structure (**SK-25**) was confirmed by spectroscopic techniques. The characterization data for the 4-(pyridin-4-yl)-6-(thiophen-2-yl) pyrimidin-2(1H)-one (**SK-25**) is as follows: Color and state: Light brown powder; Yield-88 %; mp: 275−276 °C; ^{1}H NMR (DMSO-d_6, 300 MHz, δ, TMS = 0): 8.73 (2H, *d*, J = 4 Hz), 8.19 (1H, *d*, *J* = 4 Hz), 8.00 (2H, *bs*), 7.83 (2H, *m*), 7.23 (1H, *dd*, *J* = 2 and 4 Hz) ; ^{13}C NMR ($CDCl_3$, 75 MHz, δ, TMS = 0): 164.7, 163.4, 152.4, 149.9, 135.5, 134.3, 132.6, 129.7, 128.8, 128.4, 127.6, 124.3, 106.4; Anal. Calcd. for $C_{13}H_9N_3OS$: C, 61.16; H, 3.55; N, 16.46; S, 12.56 Found: C, 60.97; H, 3.66; N, 16.09; S, 12.44 [28,30].

https://doi.org/10.1016/j.heliyon.2018.e00661

2.3. Bioassays

2.3.1. In vivo antitumour activity

All animal experiments used in this study were approved by the Institutional Animal Ethical Committee, Guru Nanak Dev University, Amritsar, Punjab, India (approval No 226/CPCSEA). The Swiss albino mice weighed in the range of (18−23 g) were housed and maintained under the controlled conditions at a temperature of (23 ± 2 °C), relative humidity (50−60 %). The room was ventilated with 100% fresh air. Animals were fed with standard pellet diet (M/s Ashirwad Industries, Chandigarh, India) and autoclaved water was provided *ad libitum*. The studies for *in vivo* anticancer activities were conducted according to the guidelines issued by National Cancer Institute (NCI) [31].

2.3.2. Ehrlich ascites carcinoma (EAC)

The *in vivo* anticancer assay was performed as earlier reported against EAC model [31]. EAC (1×10^7) cells were collected from 8-10 days old Swiss albino mice having an old ascitic tumour and injected intraperitoneally on day zero (Table 2). On day 1, all animals were randomized and estranged into four groups. **SK-25** was administered i.p. in a dose of 20 mg/kg and 30 mg/kg to Group I and Group II from days 1−9. Similarly, a positive control (Group III) was administered 5- fluorouracil (5-FU, 20 mg/kg i.p) and normal saline (0.2 ml, i.p., Group IV). On day 13, all animals were sacrificed and percentage tumour growth inhibition was calculated.

2.3.3. Ehrlich tumours (Solid) and sarcoma-180 (Solid)

EAC 1×10^7 cells were collected from the peritoneal cavity of animals having tumour and injected intramuscularly in the right thigh of all animals on day 0. The next day, all animals were randomized and alienated into four groups: Group I and Group II received **SK-25** (20 mg/kg and 30 mg/kg), Group III received positive control (5-FU) and Group IV received (normal saline) for 9 consecutive days. On day 13, all animals were sacrificed, and average tumour weight was calculated.

Table 2. Effect of **SK-25** on body weight of mice in Ehrlich ascites carcinoma assay.

Treatment	Dose (mg/kg/i.p)	Average body weight (g)			
		Day 1st	Day 5th	Day 9th	Day 12th
Control	0.2 ml N.S. i.p	22.16	23.52	24.31	26.42
SK-25	20 mg/kg/i.p	21.12	22.14	23.26	23.15
SK-25	30 mg/kg/i.p	21.23	22.16	23.17	23.14
5-FU	20 mg/kg/i.p	21.18	22.43	22.65	22.12

https://doi.org/10.1016/j.heliyon.2018.e00661

2.3.4. Hematology and biochemical analysis of EAT-bearing mice

At the end of the experiment before the day of sacrificing hematological and biochemical parameters were analyzed. Blood was withdrawn from retro-orbital plexus of the mice and aspirated in an automated hemato analyser (Sysmex 1800, Germany). The hematological parameters such as white blood cells, hemoglobin, red blood cells, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, neutrophils, basophils, lymphocytes, monocytes and esnophiles were analyzed using the automated hemato analyzer. For assessment of the biochemical analysis, blood samples were centrifuged at 1000 g for 10 min at 4 °C. Plasma samples were assayed for the determination of all biochemical parameters like glucose, serum glutamic oxaloacetic transaminase, serum glutamic-pyruvic transaminase, alkaline phosphate, urea, uric acid, creatinin, total proteins, triglyceride and cholesterol with the use of automated biochem analyzer (Erba EM360, Japan) in the process.

2.4. Acute oral toxicity assay

SK-25 was evaluated for acute oral toxicity in mice [32, 33, 34]. Six mice in each group, weighing between 18-23 g, were divided into different groups. Seven days acclimatized mice were used. Mice of group 1 served as normal control, while the remaining four groups were kept as treated groups. After treatments, clinical signs of death, the occurrence of loose bowels, tremors, convulsions, abdominal contortions, locomotion, salivation, diarrhea and lethargy were observed every day for 14 days. The animals were estranged per cage and all mice in the same cage administered the same treatment. The body weight was recorded and food consumption was calculated daily for 14 days.

2.4.1. Administration of test item

The animals were subjected to fasting along with water overnight (16−18 hours) before dosing. Each mouse received test formulations orally by gavage. Animals were fed on a normal diet after 3−4 hours of dosing. The compound **SK-25** was suspended in 0.5 % xanthan gum solution in distilled water and was administered at 5, 50, 300 and 1000 mg/kg to animals of groups 2, 3, 4 and 5, respectively. Animals of normal control were administered only vehicle.

2.5. Observational and gross pathological study

The animals of all groups were checked for mortality and any toxic symptoms at two-hourly intervals for up to 24 hours. A case side clinical examination was noted for another 13 days which included any alteration in mucous membrane, eyes, skin and fur, central nervous system, respiratory patterns, somatomotor activity and

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behavior pattern and other responses, e.g. lachrymation, etc. Particular observations, e.g. loose bowels, convulsions, tremors, salivation, laziness, sleep and coma were also recorded [32].

2.6. Assessment of adverse effects

2.6.1. Locomotor activity

The impulsive motor activity was evaluated using **SK-25** with actophotometer [35]. Each mouse was placed in a square bunged field arena (30 cm × 30 cm × 30 cm) fitted with six photocells. The movement (the number of light-beam crossings) of the animal interrupts a beam of light falling on a photocell, at which a count was recorded and displayed digitally. Each animal was placed individually in the actophotometer after 30 min before and after administration of test compounds for 5 min. Subsequently, the animals were divided into five groups, each consisting of six animals. Scores (locomotor activities) were expressed as total counts of photobeam interruption for 5 min. Animals of group 1 received vehicle only and groups 2, 3, 4, and 5 were administered **SK-25** at dose of 5, 50, 300 and 1000 mg/kg respectively. The difference in the activity, before and after drug administration, was noted. The percentage decrease in motor activity was calculated.

2.6.2. Rotarod test

The effect of **SK-25** on motor coordination and grip strength were assessed using rotarod apparatus as previously reported [36]. In brief, before the start of the experiment, animals were subjected to training using rotarod (3.7 cm in diameter, 10 rpm) waiting that they could stay on for 60 s without falling. Rotarod test was performed for 5 min after 30 min of treatment.

2.6.3. Assessment of body weight and relative organ weight

In addition to sighting analysis, body weights were also recorded. The animals were sacrificed and were subjected to thorough necropsy inspection of the exterior of the whole body and vital organs [37, 38]. Weights of organs like kidney, liver, heart, spleen, brain and lungs were recorded and the relative weight of every organ was determined after 14 days using the given formula:

$$ROW = (OW/BW) \times 100$$

where; ROW = Relative Organ Weight

OW = Organ Weight

BW = Body Weight

https://doi.org/10.1016/j.heliyon.2018.e00661

2.6.4. Histopathological study

After determining the weight, specific organs like liver, kidney and hearts to be fixed in 10% buffered formalin solution for 24 h, dehydrated in different grades of alcohol (70, 90, 95, and 100%). Tissues were entrenched in paraffin wax, sliced into 4−5 μm wide sections, and subjected to hematoxylin-eosin staining for photomicroscopic examinations using a phase contrast fluorescent microscope.

2.6.5. Statistical analysis

Comparisons were made among control and test groups using Student's t-test. Values are represented as mean ± S.E.M. (n = 10 for control group, n = 7 for test group). $^{***}P < 0.001$ for each analysis.

2.6.6. Computer-based predictions

The low energy 3D model of **SK-25** was generated using MOE [39]. The chemical structure file was further prepared by the LigPrep tool of Maestro [40, 41]. Physicochemical properties related to drug metabolism and pharmacokinetics were predicted using QikProp [42].

3. Results

3.1. *In vivo* antitumour efficacy

3.1.1. Effect of SK-25 on EAC and Ehrlich tumour (solid)

SK-25 was evaluated for *in vivo* efficacy in EAC and Ehrlich tumour (solid) models were employed [31]. Moreover, no loss of weight was observed in the treated group of animals (Table 2). **SK-25** revealed total growth inhibition of 91.56 % and 94.71 % (Table 3) in EAC and 38.64 % and 59.06 % (Table 4) in Ehrlich tumour (solid) at 20 mg/kg/i.p. and 30 mg/kg/i.p. respectively. **SK-25** caused significant inhibition of

Table 3. *In vivo* anticancer activity of **SK-25** on against EAC assay.

Ehrlich ascites carcinoma (EAC)							
Treatment	**Dose mg/kg/i.p**	**Day 12**					
		Av. volume of ascitic fluid (ml)	**Av. weight of ascitic fluid (g)**	**Av. no. of tumor cells (× 10^7)**	**% Tumor cell growth**	**% Tumor growth inhibition**	**Mortality**
Control	0.2 ml N.S.	8.02 ± 1.42	7.9 ± 1.4	168.3 ± 34.5	100	–	0/10
SK-25	20	1.74 ± 0.03	1.7 ± 0.08	14.2 ± 1.8***	8.43	91.56	0/7
SK-25	30	0.70 ± 0.08	0.6 ± 0.08	8.90 ± 1.6***	5.28	94.71	0/7
5-FU	20	0.22 ± 0.07	0.11 ± 0.05	3.57 ± 0.52***	2.13	97.87	0/7

***P < 0.001.

https://doi.org/10.1016/j.heliyon.2018.e00661

Table 4. *In vivo* anticancer activity of **SK-25** against Ehrlich tumour (Solid).

Ehrlich tumor (solid)								
Treatment	**Dose (mg/kg/i.p.)**	**Average body weight (g)**						
		Day 1st	**Day 5th**	**Day 9th**	**Day 13th**	**Av. tumor weights (mg)**	**% Tumor growth inhibition**	**Mortality**
Control	0.2 ml N.S.	21.86	22.52	23.91	25.42	1173.25 ± 88.28	–	0/10
SK-25	20	21.31	22.37	23.46	23.59	719.85 ± 62.35***	38.64	0/7
SK-25	30	21.45	22.62	23.03	22.84	480.28 ± 72.04***	59.06	0/7
5-FU	22	21.11	22.27	22.41	22.49	597.92 ± 89.1***	49.03	0/7

***P < 0.001.

tumour growth in EAC and solid tumour models of mice (Fig. 2). **SK-25** was shown to be non-toxic at all the tested doses and no mortality was observed.

3.1.2. Effect of SK-25 on sarcoma-180 (Solid) model

Efficacy of **SK-25** is determined using the sarcoma-180 (Solid) model. **SK-25** produced 32.90 % and 45.68 % inhibition of tumour growth (Table S1, Supplementary data) in sarcoma-180 (Solid) at 20 mg/kg/i.p. and 30 mg/kg/i.p. respectively. Images were taken by the digital camera (Fig. 3). Interestingly, it was found that **SK-25** did not show any mortality (0/7) at all tested doses. The tumour weight of each animal was calculated using the following formula:

$$\text{Tumour weight(mg)} = \frac{\text{Length(mm)} \times [\text{width(mm)}]^2}{2}$$

3.1.3. Hematology and biochemical analysis EAT and sarcoma-180 (solid) tumour-bearing mice

The outcomes of blood analysis and serum biochemical parameters are in normal ranges which revealed that **SK-25** is non-toxic at both the doses 20 mg/kg and 30

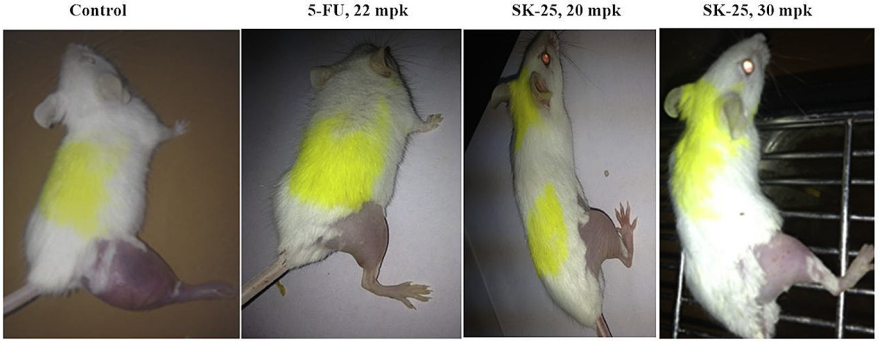

Fig. 2. Anticancer activity of **SK-25** in Ehrlich tumor solid model.

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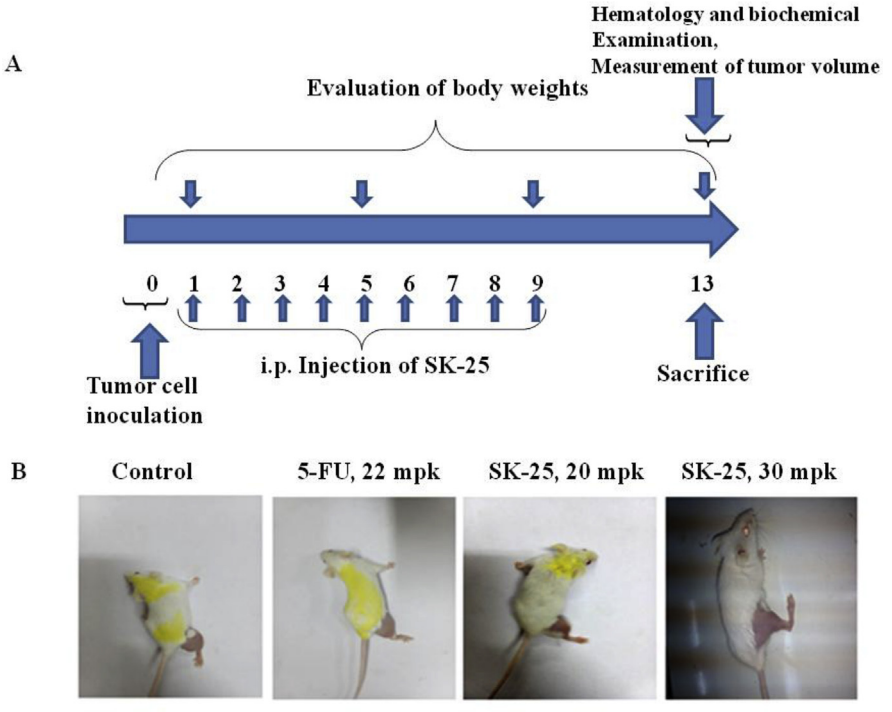

Fig. 3. Evaluation of *in vivo* anti-cancer activity of **SK-25** in Sarcoma-180 (solid) models. (A) Schematic representation of experimental plan, (B) Images of Ehrlich ascites tumor mice following treatment at 13 day.

mg/kg. No significant changes were observed when compared with positive and normal controls. The results are shown in Tables S2 and S3 (Supplementary data).

3.2. Assessment of adverse effects

3.2.1. Acute oral toxicity assay

The molecule **SK-25** was additionally analyzed for *in vivo* acute toxicity [32, 33]. The observation of treated animals over the next14 days showed no adverse effects of treatment and no signs of morbidity or mortality. No physical sign of toxicity was evidenced by behavioral changes and abnormal breathing or alterations in food

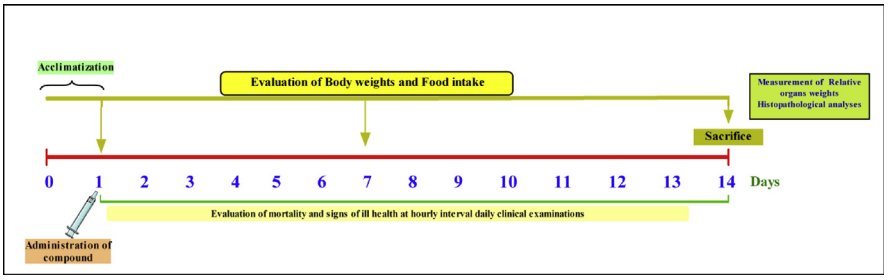

Fig. 4. Schematic representation of the experimental design for *in vivo* acute oral toxicity of **SK-25**.

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intake and modifications in body weight were observed. Overall, the study showed that compound **SK-25** was well tolerated by the Swiss-albino mice for maximum dose level of 1000 mg/kg p.o. A schematic representation of the experimental design of acute toxicity of **SK-25** is depicted in Fig. 4.

3.2.2. Observational and gross pathological analysis

All the animals were frequently observed for mortality and any toxic symptoms at two-hourly intervals for up to 24 hours. A case side clinical examination was noted for another 13 days, there was no significant alteration in mucous membrane, eyes, skin and fur, central nervous system, respiratory patterns, somatomotor activity and behaviour pattern, the occurrence of tremors, convulsions, abdominal contortions and other responses, e.g. lachrymation, etc [32]. Particular observations like loose bowels, convulsions, tremors, salivation, laziness, sleep and coma were also shown in Table S4 (Supplementary data).

3.2.3. Actophotometer test

Locomotor activity was assessed using actophotometer. No physical sign of toxicity was evidenced by behavioral changes. It was found that there is no significant decrease in locomotor activity with **SK-25** at all the tested doses such as 5, 50, 300 and 1000 mg/kg respectively when compared with vehicle-treated control group (normal saline) (Table 5).

3.2.4. Rotarod test

The mice were observed for behavioral changes and mortality within 24 h. The effect of **SK-25** was evaluated on the motor performance of the animals. The compound did not show any change in spontaneous locomotion when compared with vehicle-treated control group (Table 5).

Table 5. Effect of **SK-25** on assessment of behavioral effects in acute toxicity assay.

Treatment/dose (mg/kg)	Acute toxicity assay	
	Locomotor activity counts/5 min	Retention time/latency to fall
Vehicle control	389.11 ± 21.19	299.12 ± 18.09
SK-25 (5 mg/kg)	385.05 ± 19.89	297.75 ± 22.09
SK-25 (50 mg/kg)	380.54 ± 22.15	298.50 ± 21.71
SK-25 (300 mg/kg)	378.34 ± 23.11	295.22 ± 26.42
SK-25 (1000 mg/kg)	378.88 ± 23.32	294.72 ± 28.10

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3.2.5. Body weight and mortality

In adding to sighting study, body weights and mortality were also recorded (Table S5, Supplementary data). However, no alteration in food consumption and no significant reduction of the body weights were observed, when compared with the control group.

3.2.6. Relative organ weight

After the animals were sacrificed and subjected to detailed necropsy assessment of the peripheral surface of the body for any gross pathological changes, all animals were observed for toxic signs and any pre-terminal deaths daily (Table S6, Supplementary data). In all the treated groups, there were no considerable changes in relative organ weight of vital organs like liver, heart, kidney, spleen, brain and lungs when compared with control group depicted in (Fig. 5) [37].

3.2.7. Histopathological analyses of vital organs

No apparent changes in histological examinations were found in all organs at a dose of 1000 mg/kg as compared with normal control groups (Fig. 6). Findings confirmed no signs of toxicity, such as reduction in body weight, organ weight, and mortality at a dose of 1000 mg/kg.

3.2.8. Computer modelling and prediction

The computed DMPK predictions led to 51 computed parameters (Table S7, Supplementary data), all of which fall with the recommended range for 95% of known drugs, including Lipinski's "rule of Five" for drug-like compounds and the "Rule of Three" for Lead-like compounds.

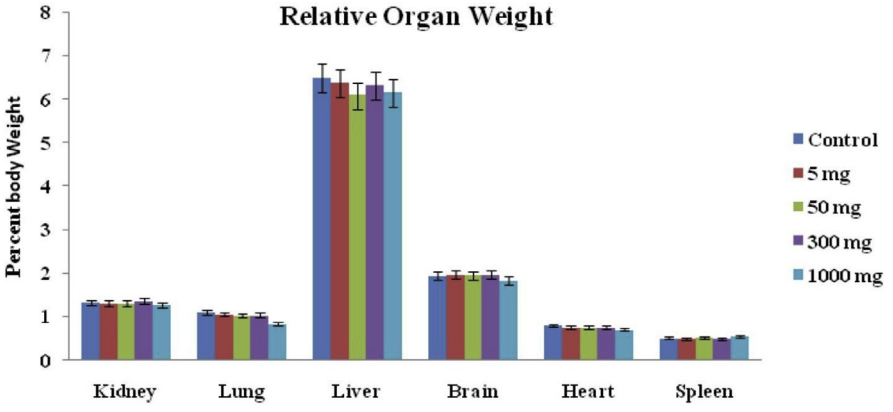

Fig. 5. Effect of **SK-25** as a single acute oral dose at 5, 50, 300 and 1000 mg/kg on relative organ weight in Swiss-albino mice.

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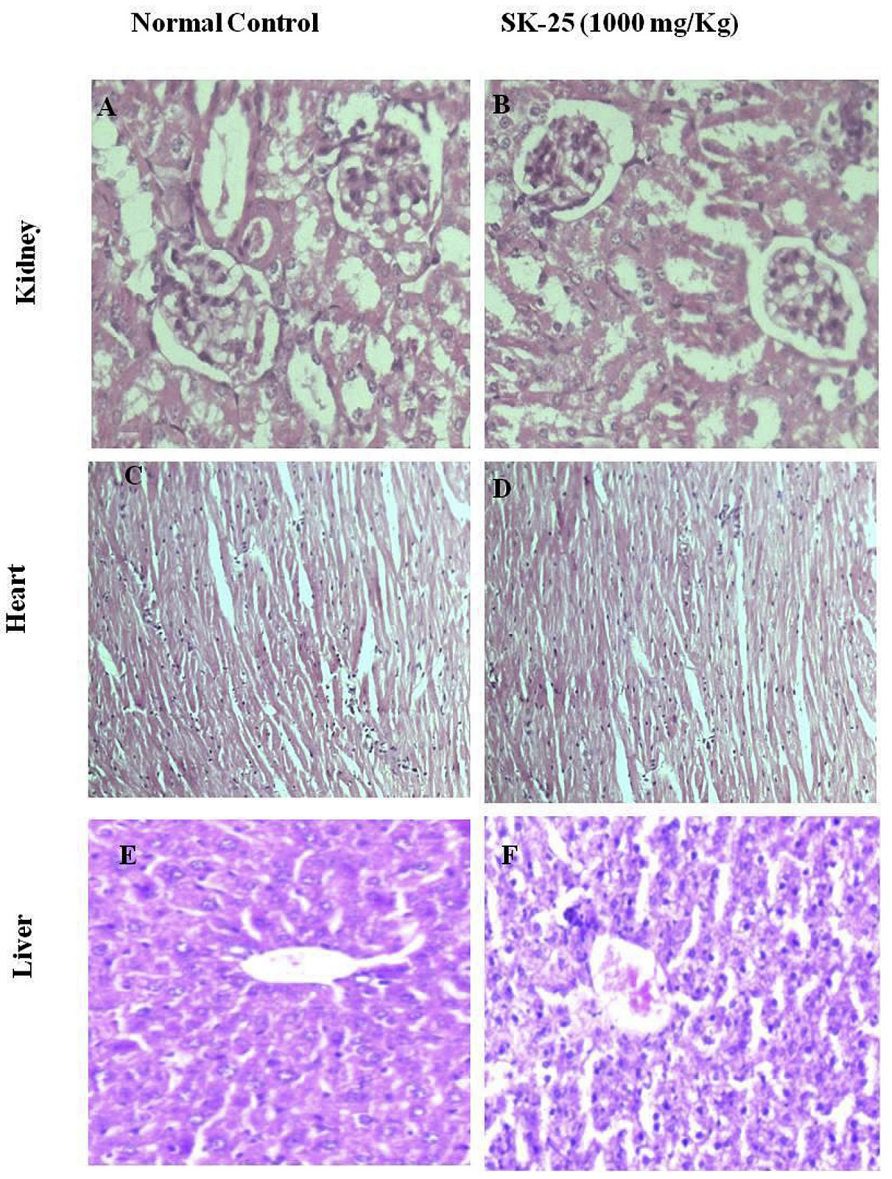

Fig. 6. Histology examination of kidney, heart and liver with single acute oral dose 1000 mg/kg of **SK-25**. The images shown here are magnified at × 20 and scale bars, 100 μm.

4. Discussion

The search for safe and potent anticancer chemotherapeutic agents with natural product scaffolds, e.g. chalcones, has been a hot topic [43, 44]. The compound under investigation was recently synthesized by a microwave-assisted multicomponent strategy and proven to be an antiproliferative agent [28, 45, 46]. In our previous study, the cytotoxicity of **SK-25** was evaluated against four different human cancer cell lines, *viz*. MiaPaCa-2 (pancreatic cancer), PC-3 (prostate cancer), A-549 (lung cancer) and HCT-116 (colon cancer). The compound had exhibited a percentage

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inhibition of 93 % and IC_{50} value of 1.95 μM towards MiaPaCa-2 malignant cells. Apoptosis was confirmed by annexin V/PI binding assay, which is a hallmark of cancer [47]. Results demonstrate a dose-dependent boost in the number of apoptotic cells in MiaPaCa-2 malignant cell lines. One of the most remarkable features of the designed compound was the selectivity displayed by the synthesized pyrimidones. The compound was selectively active against MiaPaCa-2 cell line whereas A-549 and PC-3 were resistant against the experience of test molecules. **SK-25** possessing heteroaryl rings at both 4 and 6 locations where the most powerful agent cause percentage inhibition of 93 % and IC_{50} value 1.95 μM against MiaPaCa-2 cell lines. This is because of hydrogen bonding capacity of heteroatom. **SK-25** was one of the most effective inhibitors because high aromatic character credited due to the incorporation of heterocyclic rings.

A detailed mechanistic investigation was further carried out using **SK-25** in MiaPaCa-2 cell lines. The molecule induced apoptosis, which is revealed by 4′,6-diamidino-2-phenylindole (DAPI) staining and phase contrast microscopy. Loss of mitochondrial membrane potential has been measured as an indicator of cell death. **SK-25** caused significant reduction of mitochondrial membrane potential. Mitochondrial membrane potential (MMP) loss is an early apoptotic incident and smashed mitochondria impart numerous signals to downwards that initiate inherent apoptotic death signals in cells and cells treated with compound were displaying cell cycle arrest in G0/G1 phase, which established the apoptotic capability of the molecule. The molecule **SK-25** exhibited 30.33 % arrest of the apoptotic population in test animals treated with 20 μM of **SK-25** [28]. Within the series of previously synthesized and tested chalcones possessing diheteroaryl rings additionally evaluated for cell death mechanism. Various experiments such as DAPI staining, Phase contrast microscopy, measurement of MMP loss and cell cycle analysis were performed to gain mechanistic insights. It was observed that **SK-25** caused an apoptotic induction to 30.33 % was observed on treatment at 20 μM as compared with the control sample [28].

A report of extensive investigations on chalcones shows that the presence of two aryl nuclei increases the anti-tumour prospectives [45, 46]. Thus, investigations on constrained chalcone analogues remains an area of pharmacological interest, offering immense scope for investigations on this privileged class of tubulin inhibitors. The remarkable anti-cancer potential of pyrimidine/ones, as evidenced by the number of research articles further motivated us to utilise it as a constraint for attaining a rigid arrangement of the two aromatic rings [28]. Keeping in view the significant anti-cancer potential of constrained chalcone analogues, the present study involves *in vivo* antitumour efficacy of **SK-25** on EAT (solid), Sarcoma 180 (solid) tumour and Ehrlich ascites for proving the anticancer potential of the synthesized chalcone *in vivo*.

https://doi.org/10.1016/j.heliyon.2018.e00661

In recent years, it happens to be more and more evident that the innovation of novel drugs alone is not satisfactory to ensure their evolution in drug development. Exhilarating experimental results obtained *in vitro* are very frequently followed by disappointing results from *in vivo* studies. The foremost reasons for disappointment include the deficient concentration of drug at the target site, side effects, denied specificity, higher incidence of drug resistance to cancer cells. Consequently, the current research was snooping out the *in vivo* anti-cancer potential of **SK-25** in an experimental tumour model. To investigate the potential of **SK-25** for increased therapeutic benefit against cancer, *in vivo* antitumour activity was dogged. The molecule **SK-25** was administered to tumour-bearing mice through intra-peritoneal route and it noticeably inhibited 45−95% tumour growth in all investigational tumour models.

Toxicological scrutiny often helps to drive a decision for a novel chemical entity for its clinical use as a safe and effective candidate [48, 49, 50, 51, 52]. Moreover, no mortality was observed and the tested animals did not show any stern adverse effects. Moreover, 5-FU has various adverse effects in current therapy and shows lesser effects than **SK-25**. The results revealed that compound **SK-25** had more strapping effects in ascites tumour than solid tumours. Thus, **SK-25** could be used in the treatment of non-solid tumours in future. In this report, **SK-25** has been shown to be non-toxic and inhibit tumour growth in all tumours models. Since there is no mortality throughout the experiment. Moreover, no changes were observed during hematological and biochemical toxicity after treatment with **SK-25** in sarcoma-180 (solid) and EAC (solid) tumour models which make it as a plausible choice of treatment, therapeutically effective and safe. Similarly, no mortality and signs of toxicity like reduction in body weight, food intake, relative organ weight, gross pathology of vital organs during treatment with different doses as compared with control in acute toxicity study. Moreover, no noticeable changes observed in histological examinations of major tissues when compared to normal control. Despite, the promising anti-cancer efficacy, **SK-25** was also found to be safe up to the dose of 1000 mg/kg. Therefore, **SK-25** could be further considered as a potential anti-cancer drug candidate. Nevertheless, sub-acute and chronic toxicity analysis should be done to search for any unfavourable impact on the repetitive administration of compound **SK-25** for its future development. Molecular modelling was carried out on **SK-25** as described in the Materials and methods section. The molecular descriptors calculated were those related to the prediction of DMPK/ADMET properties, e.g. brain-blood partition coefficient, aqueous solubility, serum protein binding, number of likely metabolic reactions, Caco-2 cell permeability, solvent accessible surface area, blockage of human-ether-a-go-go potassium ion (HERG K^+) channels and the total volume of the molecule enclosed by the solvent accessible surface area, beside the usual Lipinski descriptors (molecular weight, MW, the n-octanol-water partition coefficient, log P, number of H-bond donors and acceptors, HBD and HBA) [53]. The bioavailability of a compound often depends on processes like

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absorption and first-pass metabolism in the liver [54]. Meanwhile, absorption depends on the solubility and permeability of the compound, as well as interactions with transporters and metabolizing enzymes in the intestinal walls. The computed parameters which were are often used to evaluate oral absorption are the predicted aqueous solubility (QPlogS) the conformation-independent predicted the predicted qualitative human oral absorption (HumanOralAbsorption), the predicted % human oral absorption and compliance to Lipinski's famous "Rule of Five" (ro5) [55]. This is because the size of a molecule and its ability to create H-bonds, as well as its lipophilicity, shape and flexibility are important factors which determine its permeability. In particular, molecular flexibility depends on the number of rotatable bonds (#rotor), which has been shown to influence bioavailability in rats [56]. The blood-brain barrier (BBB) partition coefficients (QPlogBB) were computed and used as a predictor for access to the central nervous system (CNS). Madin−Darby canine kidney (MDCK) monolayers, are widely used to make oral absorption estimates, the reason being that these cells also express transporter proteins, but only express very low levels of metabolizing enzymes [55]. MDCK penetration predictions are used as additional criteria to predict BBB penetration. Thus, computed MDCK cell permeability values would be considered as approximations for the BBB permeability (for non-active transport). Besides, how efficient a drug is may be affected by its binding to plasma proteins, e.g. human serum albumin, lipoprotein, glycoprotein, α-, β'- and γ-globulins. Drug binding to plasma proteins greatly reduces the quantity of the drug available for general blood circulation. The less bound a drug is, the more efficiently it can traverse or diffuse across cell membranes. Hence, predictions of drug binding to plasma-protein could be estimated binding to human serum albumin; the QPlogKhsa parameter (computed to range from -1.5 to 1.5 for 95% of known drugs). Human ether-a-go-go-related gene (HERG) encodes a potassium ion (K^+) channel that is implicated in fatal arrhythmia [57]. The HERG K^+ channel contributes to the electrical activity of the heart, thus it is a molecular target responsible for the cardiac toxicity of a wide range of therapeutic drugs [58]. HERG has also been associated with modulating the functions of some cells of the nervous system and with establishing and maintaining cancer-like features in leukemic cells [59]. The results for 51 relevant descriptors have been shown in Table S7 (Supplementary Data), showing that all 51 computed parameters fall within the recommended range for 95% of known drugs.

5. Conclusions

The well-established cell-killing potential of **SK-25** motivated us to explore the anticancer potential of 4,6-diaryl pyrimidones. **SK-25** displayed noteworthy anti-cancer effects both from *in vitro* and *in vivo* studies. The present study describes the anticancer potential of **SK-25** through both solid tumours and does not show any sign

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of toxicity such as mortality, reduction in body weight, changes in hematological and biochemical toxicity studies in the tested animals. Furthermore, **SK-25** was found to be safe up to the dose of 1000 mg/kg. In addition, the acute oral toxicity of **SK-25** was also performed as per OECD guidelines No 423. Molecular modelling studies and *in silico* predictions also showed that the compounds could have an interested DMPK profile, since all 51 computed parameters fall within the accepted range for approved (marketed) drugs. This compound was observed to be most dynamic and potent with an IC_{50} value of 1.95 μM against MiaPaCa-2 cell lines and findings of the optimized lead compound have been provided. It could be concluded that **SK-25** is safe and moderate anti-tumour lead molecule along with anti-proliferative and apoptosis-inducing properties. Nevertheless, it has potential as a good candidate in future for treatment of cancer.

Declarations

Author contribution statement

Dinesh Kumar: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Pooja Sharma: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Kunal Nepali, Girish Mahajan, Mubashir J. Mintoo, Amarinder Singh: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Gurpreet Singh: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Dilip M. Mondhe: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Gurdarshan Singh: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Subheet K. Jain, Girish K. Gupta, Fidele Ntie-Kang: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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