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European Journal of Pharmaceutical Sciences 47 (2012) 988–995

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Anticancer activity, toxicity and pharmacokinetic profile of an indanone derivative

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ARTICLE INFO

Article history:
Received 22 May 2012
Received in revised form 17 July 2012
Accepted 20 August 2012
Available online 24 September 2012

Keywords:
Gallic acid
Indanone
In vivo anticancer activity
Tubulin polymerisation inhibition
Acute oral toxicity
Pharmacokinetics

ABSTRACT

The present study describes anticancer effect of gallic acid based indanone derivative (1). Indanone 1 exhibited *in vivo* anticancer activity against Erhlich ascites carcinoma in Swiss albino mice by inhibiting tumor growth by 54.3% at 50 mg/kg b.wt. It showed antitubulin effect by inhibiting tubulin polymerase enzyme. In cell cycle analysis, it inhibited G2/M phase and induced apoptosis. It significantly suppressed VEGF-R1, VEGF-R2 and HIF-α in human breast cancer MCF-7 cells, thus exhibiting antiangiogenic activity. In acute oral toxicity, indanone 1 was well tolerated and was found to be non-toxic up to 1000 mg/kg b.wt. in Swiss albino mice. Pharmacokinetic studies in rabbits revealed rate of absorption, half life, volume of distribution with high plasma and blood clearance after *i.v.* administration. Indanone 1, is a safe and moderately active anticancer agent.

1. Introduction

Cancer is a leading cause of death worldwide accounting for 7.6 million deaths in 2008 and is projected for a continuous rise, with an estimated 11 million deaths in 2030 (WHO, 2012). It is the second most common cause of death and accounts for 13% of all death in humans presently. Cancer prevalence in India is around 2.5 million with over 0.8 million new cases and 0.5 million deaths each year. Cancer killed 556,400 people across the country in 2010 (Dikshit et al., 2012). While great strides have been made in the treatment of cancer over the past 50 years, it continues to be a major health concern and, therefore, extensive efforts have been devoted to search for new therapeutic approaches (Shewach and Kuchta, 2009).

Natural products have historically provided new drugs against a wide variety of diseases, and cancer is certainly no exception. Indeed, the imagination of nature for developing toxic compounds of unusual structure is second to none (Cragg et al., 2009). A number of lead molecules have been achieved from plants i.e. camptothecin, podophyllotoxin, taxol, combretastatin, etc. Nature has provided the basis for the semisynthesis or total synthesis of effective new drugs inspired from the structure of these lead molecules. Some of the semisynthetic analogues like topotecan, irinotecan, etoposide, teniposide and taxotere are already in clinical use (Srivastava et al., 2005).

Gallic acid, a plant phenolic acid is abundantly available in woody perennials. It generally exists as hydrolysable tannin as well as in free form to some extent in higher plants. Being a simple phenolic acid and its abundance, it has been a building block of choice for many pharmacophores. We have been working on structural modifications of gallic acid to get various pharmacophores like naphthophenone fatty acid amides, aryl naphthofurans, steroidal chalcones etc. as possible anticancer agents (Saxena et al., 2007, 2008; Srivastava et al., 2006a, 2006b). In our previous studies, we identified 3-(3′,4′,5′-trimethoxyphenyl)-4,5,6-trimethoxy, 1-indanone (indanone 1, Fig. 1) as an anticancer lead molecule, exhibiting significant activity (IC_{50} = 2.2 μM) against MCF-7, estrogen dependent breast cancer cells in MTT assay and non-toxic to human erythrocytes in 'erythrocyte fragility test' (Saxena et al., 2008). In the present study, we have further evaluated this lead molecule for anticancer target, *in vivo* anticancer activity against Ehrlich ascites carcinoma, cell cycle analysis, angiogenesis and *in vivo* acute oral toxicity. A detailed pharmacokinetic profile of this lead molecule has also been established.

Abbreviations: CSIR, Council of Scientific and Industrial Research; MTT, (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole; EAC, Ehrlich ascites carcinoma; VEGF, vascular endothelial growth factor; HIF, hypoxia-induced factors; SGPT, serum glutamic pyruvic transaminase; SGOT, serum glutamic oxaloacetic transaminase; ICH, international conference on hormonisation; LOD, limit of detection; LOQ, limit of quantitation.

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http://dx.doi.org/10.1016/j.ejps.2012.08.013

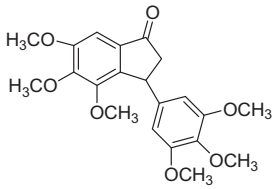

Fig. 1. Structure of indanone **1**.

2. Materials and methods

2.1. General experimental procedures

Melting point was determined in open glass capillary on E-Z Melt automated melting point apparatus, Stanford Research System, USA and was uncorrected. Reactions were performed as per standard procedures and monitored on silica gel $60F_{254}$ aluminium plates (Merck, KGaA, Germany). TLC visualization was done by inspection under UV lamp (254/365 nm) and further by spraying with a solution of 2% ceric sulphate in 10% aqueous sulphuric acid and charring at 100–110 °C for 5–10 min. Column chromatography was carried out on silica gel (60–120 mesh, Thomas Baker). Solvents were distilled prior to use. The NMR spectra were obtained on Bruker Avance-300 MHz instrument with tetramethylsilane (TMS) as an internal standard. Chemical shifts were given in δ ppm values. ^1H–^1H coupling constant (J) values are given in Hz. EI mass spectra were recorded on Perkin–Elmer TurboMass GC–MS system after dissolving the compounds in methanol. FT-IR spectra were recorded on Perkin–Elmer SpectrumBX. Purity profile of the investigating compound was done in Shimadzu LC–MS.

2.2. Enzymes and chemicals

Tubulin polymerisation assay kit was procured from Cytoskeleton USA and store at −80 °C unless used. Podophyllotoxin, colchicine, 5-fluorouracil were obtained from Sigma (USA). RPMI-1640 medium, propidium iodide (PI), DNase-free RNase, PMSF, eukaryotic protease inhibitor cocktail, penicillin, streptomycin, DMSO, PBS, L-glutamine, pyruvic acid were also purchased from Sigma Aldrich, India. Fetal bovine serum was obtained from GIBCO Invitrogen Corporation (#16000-044, lot No. 1237517) USA. Rabbit anti-human antibodies to VEGF-R1 (#2893), VEGF-R2 Fas (#2472), Rabbit anti-goat IgG-HRP (#7074) and Mouse anti-rabbit IgG-HRP (#7074) were purchased from Cell Signaling Technology Inc. USA. Mouse anti-human antibodies to Bcl-2 (#sc-7382), β-Actin (#sc-47778) and p21 (#sc-817) were from Santa Cruz Biotechnology, USA. Mouse anti-human antibodies to HIF-α (#610959) and NF-κB (#554184) were from BD Biosciences, Pharmingen, USA. Electrophoresis reagents, Protein estimation kit and protein markers were from Bio-RAD Laboratories, USA. Hyper film and ECL Plus western blotting detection kits were from Amersham Biosciences, UK. Other reagents used were of analytical grade and were available locally.

2.3. Chemical synthesis

The chemical synthesis of indanone 1 was described earlier (Saxena et al., 2008). Briefly, gallic acid was first methylated with dimethylsulphate to get 3,4,5-trimethoxybenzoic acid methyl ester. Trimethoxybenzoic acid methyl ester was reduced with lithium aluminium hydride to get 3,4,5-trimethoxybenzyl alcohol. Trimethoybenzyl alcohol was oxidized with pyridinium chlorochromate (PCC) to get 3,4,5-trimethoxybenzaldehyde. Trimethoxybenzaldehyde underwent Claisen–Schmidt reaction with 3,4,5-trimethoxyacetophenone in presence of alkali to give a hexamethoxychalcone. The hexamethoxychalcone was cyclised through Nazarov's reaction by treating with trifluoroacetic acid (TFA) in a sealed tube to get the indanone 1.

Physical data of 3-(3′,4′,5′-trimethoxyphenyl)-4,5,6-trimethoxy, 1-indanone (indanone 1).

m.p.: 107–10 °C; IR ν_{max} (KBr) cm^{-1}: 2938, 1705, 1591, 1509, 1500, 1129, EIMS: m/z (rel. int.) 389 $[M^+ + 1]$ (22%), 388 $[M^+]$ (100%); ESIMS (MeOH): 389 $[M + H]^+$, 411 $[M + Na]^+$; ^1H NMR ($CDCl_3$, 300 MHz): δ 2.58–2.66 (dd, 1H, 2-CH, J = 2.58, 19.29 Hz), 3.13–3.22 (dd, 1H, 2-CH, J = 7.98, 19.26 Hz), 3.42 (s, 3H, OCH_3), 3.78 (s, 6H, 2x OCH_3), 3.81 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 4.50–4.54 (dd, 1H, 3-CH, J = 7.95, 2.49 Hz), 6.31 (s, 2H, aromatic protons), 7.09 (s, 1H, aromatic proton); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 42.30 (C3), 47.48 (C2), 56.58 (3′-OCH_3), 56.68 (4′-OCH_3), 56.68 (5′-OCH_3), 60.44 (6-OCH_3), 61.14 (5-OCH_3), 61.14 (7-OCH_3), 100.90 (C8), 105.30 (C2′ & C6′), 132.65 (C1′), 140.42 (C3′), 140.42 (C5′), 144.39 (C9′), 149.21 (C4′), 150.89 (C4), 153.85 (C5), 153.85 (C7), 155.45 (C6), 205.08 (C1). HRMS (ESI+): 389.15763 $[M + H]^+$ for $C_{21}H_{25}O_7$. HPLC purity: 98.2% (area normalization method).

2.4. Tubulin polymerisation inhibition assay

The tubulin polymerization experiment was performed using 'Cytoskeleton, USA-assay kit' as per reported method (Lee and Timasheff, 1977; Shelanski et al., 1973). Briefly, tubulin protein (3 mg/mL) in tubulin polymerization buffer (80 mM PIPES, pH 6.9, 2 mM MgCl2, 0.5 mM EGTA, 1 mM GTP and 15% glycerol) was placed in pre-warmed 96-well microtiter plates at 37 °C in the presence of test compounds with variable concentrations. All the test compounds were mixed well and polymerization was monitored kinetically at 340 nm every min for 1 h using Spectramax plate reader. The IC_{50} value was determined from dose-dependent analysis and is defined as the concentration that inhibits the rate of polymerization by 50%. Podophyllotoxin was used as standard inhibitor of tubulin polymerase and DMSO as negative control.

2.5. DNA content and cell cycle phase distribution

MCF-7 cells (1×10^6/3 mL medium/6 well plate) treated with indicated doses of indanones 1, were collected, washed in PBS, fixed in 70% cold ethanol and placed at −20 °C overnight. Cells were washed with PBS, subjected to RNase digestion (400 μg/mL) at 37 °C for 45 min. Finally, cells were incubated with propidium iodide (10 μg/mL) for 30 min and analyzed immediately on flow cytometer FACS Calibur (Becton Dickinson, USA). The data were collected in list mode on 10,000 events and illustrated in a histogram, where the 'number of cells' (counts) was plotted against the 'relative fluorescence intensity' of PI (FL-2; λem: 585 nm; red fluorescence). Resulting DNA distributions were analyzed by Modfit (Verity Software House Inc., Topsham, ME) for the proportions of cells in G_0–G_1, S-phase, and G_2-M phases of the cell cycle (Bhushan et al., 2007).

2.6. Western blotting

2.6.1. Preparation of whole cell lysate and western blot analysis

Cells (3×10^6) were collected, washed with cold PBS and incubated with cold lysis buffer (50 mM Tris pH8.0, 150 mM NaCl, 5 mM EDTA, 1% v/v Nonidet P-40, 1 mM PMSF and 1% (v/v) eukaryotic protease inhibitor cocktail) for 30 min in ice. Cells were centrifuged at 12,000g for10 min at 4 °C and the supernatant was

collected as whole cell lysates for western blot analysis of various proteins.

The protein lysates prepared above were subjected to discontinuous SDS-PAGE analysis. Protein aliquots (50 μg) were resolved on SDS-PAGE and then electro transferred to PVDF membrane overnight at 4 °C at 30 V. Non-specific binding was blocked by incubation with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature. The blots were probed with respective primary antibodies for 2 h and washed three times with TBST. The blots were then incubated with horseradish peroxidase conjugated mouse or rabbit secondary antibodies for 1 h, washed again three times with TBST and signals detected using ECL plus chemiluminescence's kit on X-ray film (Bhushan et al., 2007). Protein was measured employing Bio-Rad protein assay kit using bovine serum albumin as standard.

2.7. In vivo anticancer activity against Ehrlich ascites carcinoma

The protocol described earlier (Geran et al., 1972.) was followed for studying *in vivo* anticancer activity against Ehrlich ascites carcinoma (EAC). EAC cells were collected from the peritoneal cavity of the female Swiss mice harbouring 8–10 days old ascitic tumour. 1×10^7 EAC cells were injected intraperitoneally in each of the Swiss mice selected for the experiment on day 0. The next day, animals were randomized and divided into different groups. The treatment groups contained 7 animals each and control group contained 10 animals. Three treatment groups were treated with 50 mg/kg of indanone 1, 75 mg/kg of indanone 1 and 20 mg/kg of 5-fluorouracil (positive control) intraperitoneally from day 1 to 9. The tumour bearing control group was similarly administered normal saline (0.2 ml, *i.p.*). On day 12, animals were sacrificed and ascitic fluid was collected from the peritoneal cavity of each mouse for the evaluation of tumor growth. Percent tumor growth inhibition was calculated based on the total number of tumor cells present in the peritoneal cavity as on day 12 of the experiment using the following formula.

$$\%\,\text{tumour growth inhibition} = \frac{\text{Av. No. of cells in control group} - \text{Av. No. of cells in treated group}}{\text{Av. No. of cells in control group}} \times 100$$

2.8. In vivo anticancer activity against Ehrlich tumor (solid)

Ehrlich ascites carcinoma (EAC) cells were collected from the peritoneal cavity of the male Swiss mice harbouring 8–10 days old ascitic tumour. 1×10^7 EAC cells were injected intramuscularly in the right thigh of 24 Swiss male mice selected for the experiment on day 0. The next day, animals were randomized and divided into three groups. Two treatment groups contained 7 animals each and one control group contained 10 animals. One of the treatment groups was treated with indanone 1 (100 mg/kg i/p) from day 1 to 9. Another treatment group was treated with 5-fluorouracil (22 mg/kg, i.p) from day 1 to 9 and it served as positive control. The tumor bearing control group was similarly administered normal saline (0.2 mL, *i.p.*) from day 1 to 9. On days 9 and 13, tumor bearing thigh of each animal was shaved and longest and shortest diameters of the tumor were measured with the help of Vernier calipers. Tumor weight of each animal was calculated using the following formula:

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

The percent tumor growth inhibition was calculated on day 13 by comparing the average values of treated groups with that of control group. Tumor growth in saline treated control animals was taken to be 100%.

2.9. In vivo acute oral toxicity

Acute oral toxicity of indanone 1 was carried out in Swiss albino mice. Experiment was conducted in accordance with the Organization for Economic Co-operation and Development (OECD) test guideline No 423 (1987). For the study, 20 mice (10 male and 10 female) were taken and divided into five groups comprising 2 male and 2 female mice in each group. The animals were maintained at 22 ± 5 °C with humidity control and also on an automatic dark and light cycle of 12 h. The animals were fed with the standard rat feed and provided *ad libitum* drinking water. Mice of group 1 were kept as control and animals of groups 2, 3, 4 and 5 were kept as experimental. The animals were acclimatized for 7 days in the experimental environment prior to the actual experimentation. The test compound was suspended in 0.7% carboxymethylcellulose (CMC in water) using traces of ethanol as co-solvent and was given at 5, 50, 300 and 1000 mg/kg body weight to animals of groups 2, 3, 4 and 5 respectively. Control animals received only vehicle. All the animals were sacrificed on 7th day after the experimentation.

The animals were checked for mortality and any signs of ill-health at hourly interval on the day of administration of indanone 1 and thereafter a daily general case side clinical examination was carried out throughout the experimental period. Parameters like changes in skin, mucous membrane, eyes, occurrence of secretion and excretion and also responses like lachrymation, pilo-erection, respiratory patterns, changes in gait, posture and response to handling were recorded (Allan et al., 2007). In addition to observational study, body weights were recorded and blood and serum samples were collected from all the animals on 7th day after experiment and were analysed for total RBC, WBC, differential leucoyte count, haemoglobin percentage and biochemical parameters like total cholesterol, triglycerides, creatinine, SGPT, SGOT. The animals were then sacrificed and were necropsed for any gross pathological changes. Weights of vital organs like liver, heart, kidney etc. were recorded and a portion of liver from each animal were saved and analyzed for hepatic reduced glutathione and malonaldehyde content (Chanda et al., 2009).

2.10. In Vivo pharmacokinetic studies

Adult male and female New Zealand white rabbits (~2.5 kg body weight), were acclimatized to the experimental environment before experiment. The protocols used were duly approved by Institutional animal ethics committee (IAEC). The dose of indanone 1 for rabbit was calculated from *in vivo* effective dose for rat (50 mg/kg) based on surface area and basic metabolic rate as described by Paget and Barnes, (1964). Thus, the dose calculated for rabbit was approximately 16 mg/kg body weight and was used in the pharmacokinetic study. The indanone 1 was dissolved in 12.5% cremophore EL (Sigma) in 0.9% normal saline solution and was administered intravenously to rabbit through ear vein. Blood samples (approximately 0.75 mL) were collected from other ear vein at 0, 0.08, 0.25, 0.5, 1, 2, 4, 6, 8, 12 h interval from the time of administration in heparinised tubes. The blood samples were then centrifuged at 7000*g* for 10 min at 4 °C and the plasma samples obtained were stored at −80 °C for analysis and estimations of indanone 1 concentrations.

The plasma concentrations of indanone 1 were determined by reversed-phase HPLC method using a solid phase extraction. The brief description is as follows: column: C_{18} Symmetry® analytical, 4.6 × 250 mm, 5 μm column (Waters, USA); mobile phase: acetonitrile and water (containing 0.1% AcOH (65:35, v/v); detection in

Photodiode array (PDA) at 228 nm with LC-MS specificity. The method was validated following the ICH guidelines.

2.11. Statistical analysis

Data expressed as mean or representative of one of three similar experiments unless otherwise indicated. Comparisons were made between control and treated groups unless otherwise indicated using unpaired Student's *t*-test and *p* values <0.01 was considered significant. The pharmacokinetic parameters were computed with MS Excel® (Microsoft, Seattle, WA, USA) and PK Solver 2.0 Excel Add-ins. The estimation of AUC was calculated by the trapezoidal rule. Additionally, clearance for the indanone 1 metabolite was expressed as CL/fm due to lack of information regarding what fraction of an *i.v.* dose of drug is converted to systemically available metabolite (fm).

3. Results

3.1. Chemical synthesis

The synthetic strategy was as described earlier (Saxena et al., 2008). Gallic acid was modified to indanone 1 in four synthetic steps with an overall yield of 8.3%.

3.2. Biological evaluation

3.2.1. Tubulin polymerization inhibition

Indanone 1, inhibited tubulin polymerase, moderately (IC_{50} = 1.88 μM), while podophyllotoxin, the standard antitubulin showed comparatively higher activity (IC_{50} = 0.74 μM).

3.2.2. Cell cycle analysis

Measurement of DNA content makes it possible to identify apoptotic cells, to recognize the cell cycle phase specificity and to quantitate apoptosis. Propidium iodide (PI) is a DNA binding dye and it is become highly fluorescent after binding to nuclear DNA content, which was measured through flow-cytometry.

MCF-7 cells treated with indanone 1 for 24 h exhibited concentration dependent increase in hypo diploid sub-G0 DNA fraction (apoptotic, <2nDNA, Fig. 2). The sub-G0 fraction was 1% in control cells, which increased to 58% after 100 μg/ml of indanone 1 treatment. Further, the cell cycle G2/M phase was blocked by indanone 1 at lower dose (10 μg/ml) from 12% to 17% indicating that it produced mitotic block or caused delay in cell cycle (Fig. 2). Whereas, indanone 1 at 30 and 100 μg/mL produced significant G1 arrest with no significant mitotic block. Thus, indanone 1 seems to inhibit G2/M phase at lower doses and G1 arrest at higher doses in MCF-7 cell cycle analysis.

3.2.3. Western blot analysis

Effect of indanone 1 on apoptotic and angiogenic related protein expression

Indanone 1 decreased the expression of nuclear transcription factors NF-κB and cyclin dependant kinase inhibitor p21 in a dose-dependent manner in MCF-7 cells (Fig. 3). Both these transcriptional factors are highly expressed in these cells and were accumulated in the nucleus of cells, which might have important implication in overcoming drug resistance, cell division and apoptosis. p21 is now recognized as a master regulator of cell fate in response to oxidative stress and various stimuli. It is a potent inhibitor of cyclin dependant kinases and its expression is tightly controlled by p53. Simultaneously, indanone 1 significantly down

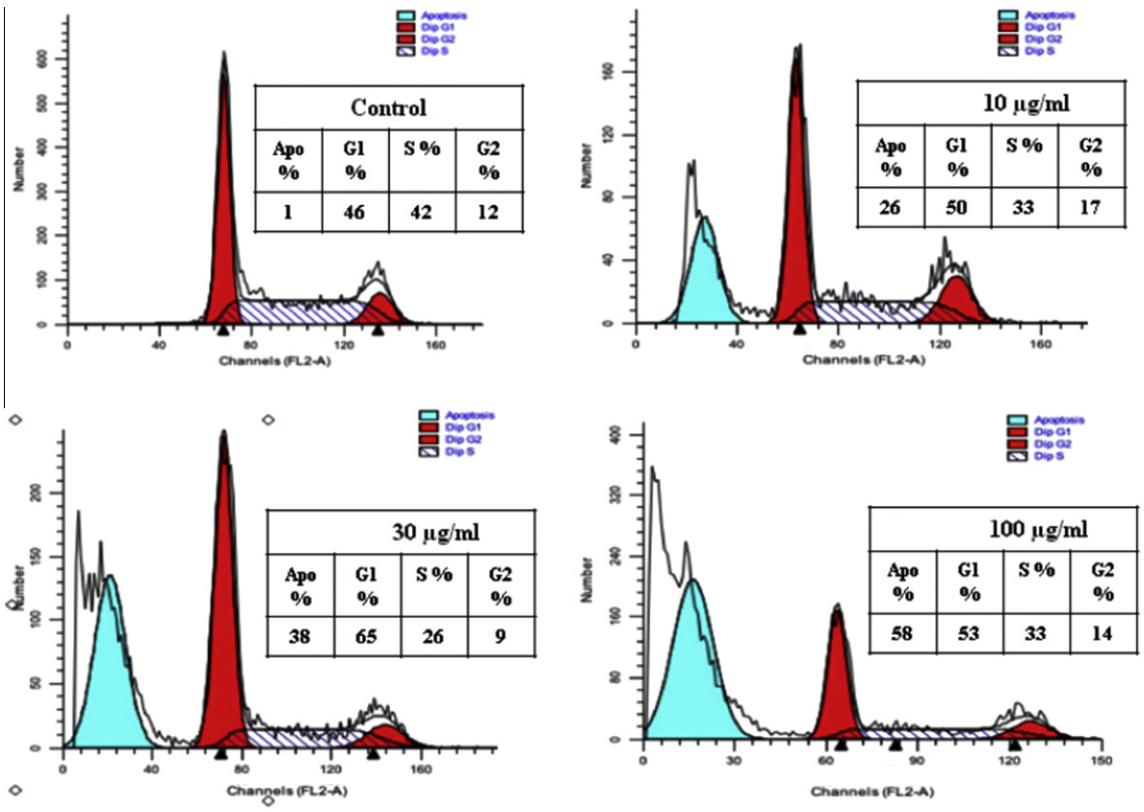

Fig. 2. DNA cell cycle analyses in indanone 1 treated MCF-7 cells. MCF-7 cells (1×10^6) were exposed to different concentrations of indanone 1 for 24 h and stained with PI to determine DNA fluorescence and cell cycle phase distribution as described in Section 2. Data were analyzed by Modfit software (Verity Software House Inc., Topsham, ME) for the proportions of different cell cycle phases. Fraction of cells from apoptotic, G1, S and G2 phases analyzed from FL2-A vs. cell counts is shown in%. Data are representative of one of three similar experiments.

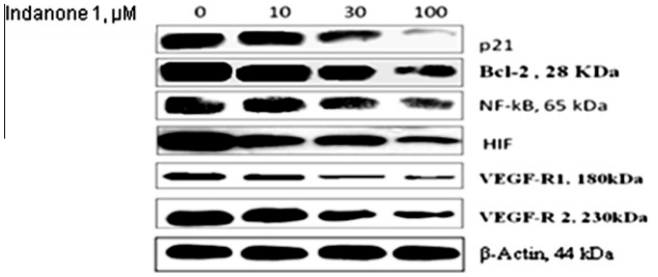

Fig. 3. Influence of indanone 1 on the expression of anti-apoptotic, transcriptional factor, cell cycle regulator and anti-angiogenic proteins. MCF-7 cells (1×10^6) were treated with indicated concentration of indanone 1 for 24 h. β-Actin was used as internal control to represent the same amount of proteins applied for SDS–PAGE. Specific antibodies were used for detection of cytochrome c, Smac/DIABLO, Bax and Bcl-2. B) Influence of TPD on the Bcl-2/Bax ratio in the mitochondrial fraction of HL-60 cells. Relative density of each band was measured as arbitrary units by Quantity One software of Bio-RAD gel documentation system (data not shown). Data are representative of one of three similar experiments. *p* values: * <0.01 compared to untreated control.

Table 1
Effect of indanone 1 on body weight of mice bearing Ehrlich ascites carcinoma.

Treatment	Dose	Body weight (g)			
		Day 1	Day 5	Day 9	Day 12
Control	0.2 ml N.S. *i/p*	23.1	25.5	27.2	28.3
Indanone 1	50 mg/kg *i/p*	20.85	22.14	22.85	23.4
Indanone 1	75 mg/kg *i/p*	20.85	22.0	22.55	23.04
5-FU	20 mg/kg *i/p*	21.57	22.14	20.42	20.71

regulated the expression of mitochondrial anti-apoptotic protein Bcl-2 in dose dependent manner which all gears up pro-apoptotic machinery (Fig. 3). Angiogenesis is a fundamental event in the process of tumor growth and transition of tumors from a dormant state to a malignant one. Indanone 1 significantly inhibited the expressions of VEGF-R1, VEGF-R2 and Hypoxia-inducible factor, HIF-α in MCF-7 cells (Fig. 3) which have pivotal role in tumor angiogenesis. Vascular endothelial growth factor receptors play a pivotal role in normal and pathologic angiogenesis. Its overexpression has been associated with tumor progression and poor prognosis in several tumor systems including breast cancer.

3.2.4. In vivo anticancer activity

Indanone 1 showed 54.30% inhibition in the growth of Ehrlich Ascites Carcinoma cells in the peritoneal cavity of experimental animals at a dose of 50 mg/kg body weight (Tables 1 and 2). Further, increase in dose to75 mg/kg body weight yielded only marginal increase in the activity to 55.83% (Tables 1 and 2). However, against solid form of the Ehrlich tumor, indanone 1 exhibited reduced level of activity (26.84% growth inhibition) at a dose of 100 mg/kg (Table 3).

3.2.5. In vivo acute oral toxicity

There were no observational changes, morbidity and mortality throughout the experimental period. Blood and serum samples upon analysis showed non-significant changes in all the parameters studied like total RBC, WBC count, differential leukocyte count, haemoglobin, serum total cholesterol, triglycerides, creatinine, SGPT and SGOT activity (Table 4 and Fig. 4). Similarly, animals on gross pathological study showed no changes in any of the organs studied including their absolute and relative weights (Fig. 5A and B).

3.2.6. Pharmacokinetic profile

The validated HPLC-PDA method was successfully applied in plasma matrix to determine the pharmacokinetic profile (Table 5, Figs. 6 and 7) of indanone 1 after single-dose intravenous administration in adult rabbits. The LOD and LLOQ in plasma were estimated to be 4.63 and 15.40 ng/mL respectively. There were no interfering peaks co-eluted with the analytes of interest and the mobile phase used for the assay provided a well defined separation ($Rs > 1.50$) between indanone 1 and plasma exogenous. Representative chromatogram is shown in Fig. 6 for plasma from a dosed rabbit (16 mg/kg indanone 1), revealing no interfering compounds. The mean plasma concentration–time profile of indanone 1 is presented in Fig. 7. Estimated non-compartmental parameters of indanone 1 are shown in Table 5. The drug reached peak plasma concentration (C_{max}) of 32.45 μg/mL and could be detected up to 4 h post dosing after which indanone was undetectable in plasma. The high clearance rate (CL) 0.54 L/h/kg was in corroboration with low $t_{1/2}$ value (1.94 h) and MRT value (2.71 h). Though the metabolites could not be specified in the present study, but possibility of partial sulphatase activity (β-glucuronide), *O*-dealkylation and hydroxylation could not be ignored. Similarly, the possibility of higher levels of the hydroxylated metabolites than of their respective glucuronide (or sulphate) conjugates could be proposed on the

Table 2
Effect of indanone 1 on against Ehrlich ascites carcinoma.

Treatment	Dose	Day 12					
		Av. volume of ascitic fluid (ml)	Av. weight of ascitic fluid (g)	Av. no. of tumor cells	% Tumour cell growth	% Tumor growth inhibition	Mortality
Control	0.2 ml N.S. *i/p*	8.99	9.05	286.95×10^7	100	–	0/10
Indanone 1	50 mg/kg *i/p*	4.14	3.92	131.12×10^7	45.7	54.30	0/7
Indanone 1	75 mg/kg *i/p*	4.00	3.86	126.72×10^7	44.17	55.83	1/7
5-FU	20 mg/kg *i/p*	0.92	0.91	15.32×10^7	5.34	94.66	0/7

Table 3
Effect of indanone 1 against Ehrlich tumor (solid).

Treatment groups	Av. body weights (g) of animals on days			Day 13		% Tumor growth inhibition	Mortality
	1	5	9	Av. body weights (g)	Av. tumor weights (mg)		
Indanone 1 (100 mg/kg *i/p*)	20.14	21.85	22.0	22.42	1398.14	26.84	0/7
5 FU (22 mg /kg *i/p*)	20.71	21.42	19.66	20.16	912.66	52.24	0/7
Control (normal saline) (0.2 ml *i/p*)	21.33	22.44	23.11	24.33	1911.16	–	0/10

Table 4
Effect of indanone 1 as a single acute oral dose on haemogram and serum biochemical parameters in Swiss albino mice ($n = 4$).

Parameters	Indanone 1 at mg/kg body weight as a single oral dose				
	Control	5 mg/kg	50 mg/kg	300 mg/kg	1000 mg/kg
Body weight (g)	36.03 ± 0.89	31.09 ± 2.98	30.19 ± 2.12	30.82 ± 1.45	28.16 ± 0.61
Total WBC count (thousands/mm^3)	4.28 ± 0.45	5.56 ± 1.41	5.59 ± 0.85	3.80 ± 0.18	4.56 ± 0.67
Total RBC count (millions/mm^3)	8.17 ± 1.13	7.69 ± 0.74	7.32 ± 0.72	7.90 ± 0.73	7.53 ± 0.59
Haemoglobin (g/dL)	15.18 ± 0.34	13.58 ± 0.37	15.68 ± 0.87	13.95 ± 1.38	12.11 ± 0.88
SGOT (U/L)	32.40 ± 3.84	32.66 ± 2.50	32.50 ± 1.53	38.16 ± 4.58	36.38 ± 3.30
SGPT (U/L)	11.07 ± 1.84	11.23 ± 1.31	12.60 ± 1.26	10.49 ± 2.16	12.86 ± 1.71
Creatinine (mg/dL)	1.44 ± 0.14	1.31 ± 0.08	1.03 ± 0.12	1.34 ± 0.07	1.22 ± 0.05
Total serum cholesterol (mg/dL)	99.86 ± 16.85	131.22 ± 16.67	124.10 ± 8.80	113.93 ± 9.31	151.45 ± 15.72
Serum triglycerides (mg/dL)	101.01 ± 21.40	118.17 ± 17.29	129.56 ± 20.65	116.49 ± 7.20	126.47 ± 9.18
Reduced glutathione (μ mole/mg protein)	23.78 ± 4.42	27.45 ± 3.43	23.86 ± 5.75	24.53 ± 3.78	25.60 ± 2.08
Malonaldehyde (n mole/mg protein)	0.84 ± 0.10	0.90 ± 0.12	0.73 ± 0.05	0.87 ± 0.11	0.81 ± 0.04

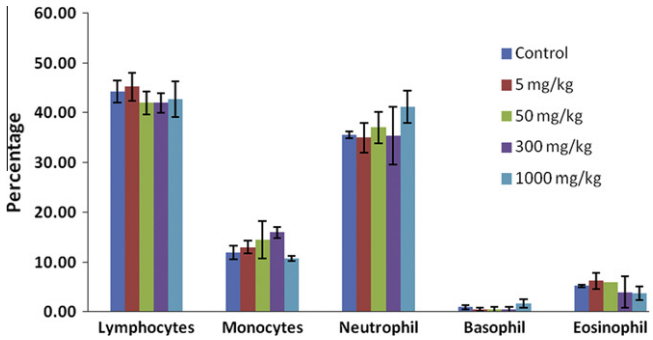

Fig. 4. Effect of indanone 1 as a single acute oral dose @ 5, 50, 300 and 1000 mg/kg body weight on differential leucocytes counts in Swiss albino mice ($n = 4$).

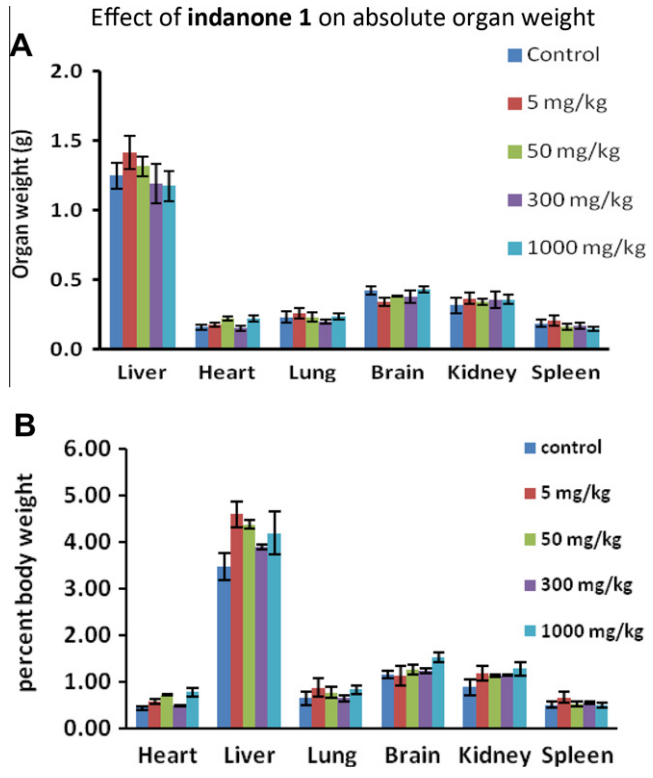

Fig. 5. (A and B): Effect of indanone 1 as a single acute oral dose at 5, 50, 300 and 1000 mg/kg body weight on absolute and relative organ weight in Swiss albino mice ($n = 4$).

Table 5
Pharmacokinetic parameters of indanone 1 in rabbits observed after *i.v.*[a] administration.

Parameter	Value
Measured dose (mg/kg)	16
$t_{1/2}$ (h)	1.94
T_{max} (h)	0.08
C_{max} (μg/mL)	32.45
AUC_{0-t} (μg/mL h)	22.62
$AUC_{0-\infty}$ (μg/mL h)	29.87
CL (L/h/kg)	0.54
CL/fm [(mg)/(μg/mL)/h][b]	0.39

Values are the mean from three animals.
[a] First measured time point following the *i.v.* bolus was 0.083 h.
[b] CL/fm = $CL_{drug} \times AUC/AUMC$.

basis of metabolism of drugs having indanone ring (Tiseo et al., 1998).

4. Discussion

In the recent past, indanocine, an indanone derivative was reported as a potent cytostatic and cytotoxic clinical investigational drug that blocks tubulin polymerisation (Leoni et al., 2000). It induces apoptotic cell death in stationary phase multidrug-resistant cancer cells (i.e. MCF-7/ADR, HL-60/ADR, MES-SA/ADR) without impairing viability of normal non-proliferative cells. Indanocine interacts with tubulin at the colchicine binding site, subsequently inhibiting and disrupting the mitotic apparatus in dividing cells (Das et al., 2009).

There are several naturally occurring tubulin polymerisation inhibitors like colchicine, podophyllotoxin, combretastatins etc. exerting antitubulin effect through the 3,4,5-trimethoxyphenyl unit (Srivastava et al., 2005). Both the polymerisation of tubulins to microtubulin and reverse process of depolymerisation are tightly controlled during mitosis, disruption of the process leads to cell cycle arrest and ultimately cell death (Wang et al., 1999). Owing to the similar structural configurations the cytotoxicity of indanone 1 against MCF-7 cells is most likely due to its antitubulin effect.

Cancer cells evade apoptosis by over-expression of several anti-apoptotic proteins like Bcl-2, p21, NF-κB. The induction of apoptosis in cancer cells is one of the approaches to limit uncontrolled cell proliferation (Ziegler and Kung, 2008), hence we investigated apoptotic effect of indanone 1 in MCF-7 cells. In our study, we observed that indanone 1 induces apoptosis by inhibiting G2/M phase of cell cycle as well as cell cycle regulator p21 in human breast cancer MCF-7 cells. Thus, by inhibiting the p21 level by indanone 1 sensitizes the MCF-7 cells to undergo apoptosis via inhibiting the mitochondrial anti-apoptotic protein Bcl-2 as well as nuclear

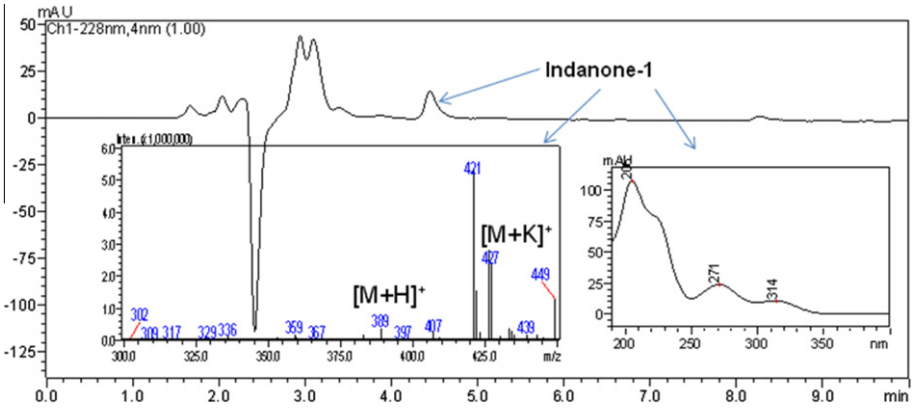

Fig. 6. Representative chromatograms at 228 nm of plasma.

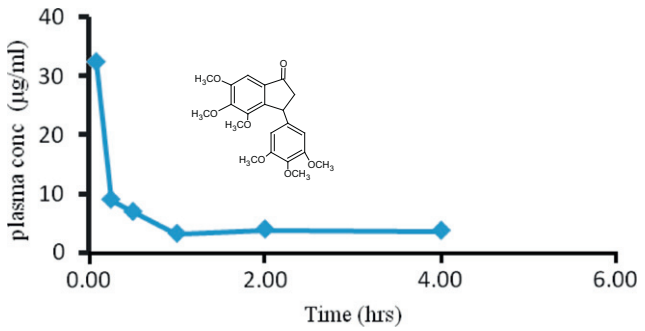

Fig. 7. Mean plasma concentration–time profiles of indanone 1 (16 mg/kg) after *i.v.* dose in rabbit ($n = 3$).

transcriptional factor NF-κB. p21 is a potent cyclin-dependent kinase inhibitor (CKI). The p21 (CIP1/WAF1) protein binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and thus functions as a regulator of cell cycle progression at G1. The expression of this gene is tightly controlled by the tumor suppressor protein p53. p21 is known to be directly regulated by the tumor suppressor p53 and repression of p21 may also have an anticancer effect. Because loss of p21 usually increases sensitivity of tumor cells to apoptosis induced by different chemotherapeutic agents, small molecules that eliminate p21 expression may improve the action of anticancer drugs (Gartel and Radhakrishnan, 2005).

Tumor angiogenesis is an important process for the development of new blood vessels. This process provides nutrition and oxygen supply to tumour cells by which they invade and spread to other parts of the body. Therefore, by blocking the development of new blood vessels, researchers are hoping to cut off the tumor's supply of oxygen and nutrients, and therefore prevent tumor metastasis. There are several growth factors that promote tumor angiogenesis among these vascular endothelial growth factor (VEGF) receptors and HIF-α play a pivotal role. Indanone 1 has exhibited significant effect on this process also. It has significantly suppressed VEGF-R1, VEGF-R2 and HIF-α in human breast cancer MCF-7 cells. HIF1 regulates the transcription of a broad range of genes that facilitate responses to the hypoxic environment, including genes regulating angiogenesis, erythropoiesis, cell cycle, metabolism and apoptosis (Gunton et al., 2005). VEGF is a key factor of tumour angiogenesis and activation of the VEGF/VEGF-receptor (VEGFR) axis triggers multiple signaling networks that result in endothelial cell survival, mitogenesis, migration, and differentiation (Jung et al., 2005; Mirzoeva et al., 2008; Schoppmann et al., 2006). Over-expression of VEGF has been associated with tumor progression and poor prognosis in several tumor systems, including colorectal carcinoma, gastric carcinoma, pancreatic carcinoma, breast cancer, prostate cancer, lung cancer, and melanoma.

In pharmacokinetic study, the maximum concentration of indanone 1 in plasma reached within 2 min, but got eliminated at the rate of 0.39 mL/h. The residual indanone 1 concentration in plasma was very less as evidenced by AUC (0–∞) 29.89 µg/mL. The clearance for indanone 1 was 0.54 mL/h. The exact estimation of indanone 1 metabolite clearance was impossible to calculate due to lack of information regarding what fraction of an *i.v.* dose of drug is converted to systemically available metabolite. However, optional calculation (CL/fm) showed that possible metabolites were also eliminated with same rate.

In acute oral toxicity experiment indanone 1 was well tolerated by the Swiss albino mice up to the dose level of 1000 mg/kg bodyweight as a single acute oral dose. There were no significant changes in hematological and other parameters. It can be considered safe up to this dose. However, sub-acute and chronic experiments with the test drug needs to be carried out to look for any adverse effect if any on repeated exposure to the test drug indanone 1 for its future development (Ghosh, 1984.).

5. Conclusion

In conclusion, the gallic acid based indanone 1 exhibits promising anticancer activity against metastatic form of breast cancer. It inhibits breast cancer cell growth by inhibiting cell cycle in G2/M phase and cyclin dependant kinase inhibitor p21, which further overcomes the resistance to apoptosis and inhibits anti-apoptotic protein Bcl-2 and NF-κB. Simultaneously, it also inhibits angiogenesis by inhibiting the expression of VEGF and HIF-α. Therefore, indanone 1 is useful in both management and treatment of breast cancer. These results provide the basis for further in-depth drug targeted studies, while the apoptotic and anti-angiogenic potential of indanone 1 raises its use as novel anticancer agent.

Acknowledgement

The authors extend due thanks to Director CIMAP for support and encouragement.

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