

17-AAG MEDIATED TARGETING OF HSP90 LIMITS TERT ACTIVITY IN PERITONEAL SARCOMA RELATED MALIGNANT ASCITES BY DOWNREGULATING CYCLIN D1 DURING CELL CYCLE ENTRY

M. Chaklader, P. Das, J.A. Pereira, A. Law, S. Chattopadhyay, R. Chatterjee, A. Mondal, S. Law*
Stem Cell Research and Application Unit, Department of Biochemistry and Medical Biotechnology,
Calcutta School of Tropical Medicine, Kolkata-700073, West Bengal, India

Aim: Peritoneal or retro-peritoneal sarcomatosis related malignant ascites formation is a rare but serious consequence of the locoregional metastatic event. The present work aimed to study the effect of the Hsp90 inhibitor (17-AAG), an ansamycin analog, on cell cycle and DNA replication specific chaperone-clients interaction in the event of peritoneal sarcoma related malignant ascites formation in mouse model at the late stage of malignant growth. **Methods:** We administered 17-AAG, an Hsp90 inhibitor, divided doses (330 µg/kg b.w./day for first five days then next ten days with 166 µg/kg b.w./day) through intra-peritoneal route of inbred Swiss albino mice bearing full grown peritoneal malignant ascites of sarcoma-180. Our study was evaluated by peripheral blood hemogram analysis, malignant ascitic cytology, cell viability test, survival time and mitotic indexing. Furthermore, flowcytometric HSP90, TERT, CyclinD1, PCNA and GM-CSF expression analysis has been considered for special objective of the study. **Results:** Our experimental efforts reduced the aggressive proliferation of malignant ascites by drastic downregulation of TERT and cyclin D1 on the verge of cell cycle entry along with DNA replication processivity factor PCNA by directly modulating their folding machinery — heat shock protein 90. Consequently, we observed that malignant ascitic cells became error prone during the event of karyokinesis and produced micronucleus containing malignant cells with low viability. Peripheral neutrophilia due to over-expression of GM-CSF by the peritoneal malignant ascites were also controlled by the treatment with 17-AAG and overall, the treatment modality improved the median survival time. **Conclusion:** Finally we can conclude that 17AAG administration might serve as a prospective pharmacological agent for the management of peritoneal sarcoma related malignant ascites and throws light towards prolonged survival of the patients concerned.

Key Words: 17-AAG, HSP90, TERT, sarcoma-180, malignant ascites, neutrophilia.

Malignant peritoneal ascites formation from the primary neoplastic growth is a very critical and troublesome medical emergency faced by most of the medical and surgical oncologists. In the context of carcinomatosis related malignant peritoneal ascites generation is a common feature [1]. In the modern clinical practice, peritoneal or retro peritoneal sarcomatosis related malignant ascites formation and its proper management is actually less discussed issue. Peritoneal leiomyosarcoma, liposarcoma, carcinosarcoma gastrointestinal stromal tumor and primary peritoneal spindle cell sarcoma have primitive mesodermal origin and can promote peritoneal malignant ascites growth [2–5]. One unusual example of the peritoneal sarcomatosis is peritoneal Kaposi's sarcoma in HIV patient which can initiate peritoneal malignant ascites as a result of metastasis [6]. However, irrespective of the peritoneal sarcomatous origin of ascites, the main objective of our present study was two folds, 1) to understand the pathobiology of the peritoneal sarcomatosis related ascites formation and 2) to find out the proper molecular target for pharmacological management of the said pathophysiology.

In this context, very few pharmacological and surgical options are available to combat the situation properly. Management of peritoneal ascites by locoregional treatment is an attractive option as the malignant ascites remains confined to the intra-peritoneal cavity during most of their natural history. Most of the conventional routes of chemotherapy administration are either intravenous or oral which are very effective but have high risk of side effects too where loco-regional chemotherapeutic treatment can be a better option. So to bring down the unwholesome effect on non target organs, locoregional chemotherapeutic administration should be considered wherever it is possible [7–9]. In our study, we also showed the use of intraperitoneal route of chemotherapy administration to manage the experimentally induced malignant ascites.

As the drug resistance is a matter of concern along with route of drug administration, the choice of pharmacological agents and pharmacological target to be taken under consideration. The present study considers chaperone-Hsp90 which is the main pharmacological target. Additionally we also consider the status of some important Hsp90 specific clients in the event of malignant ascites formation and progression. In general, chaperones are ubiquitous, highly conserved proteins which play a major role in the evolution of most of the modern proteins [10–12]. Chaperones are vital for all kind of cells for their whole lifespan and it saves the cells from proteotoxic damages coming from the external environment. Besides the assistance of folding and refolding of protein, chaperones are

Received: April 30, 2012.

*Correspondence: E-mail: msuj2002@yahoo.co.in

Abbreviations used: GM-CSF – granulocyte macrophage-colony stimulating factor; Hsp90 – heat shock protein 90; ILS – increased life span; MI – mitotic index; MRA – malignancy related ascites; MST – median survival time; PCNA – proliferating cell nuclear antigen; 17-AAG – 17-allylamino-17-demethoxygeldanamycin; TERT – telomerase reverse transcriptase.

also helpful for the cellular remodeling by maintaining the cytoplasmic meshwork i.e. microtubular lattice [13–15]. Chaperones target newly synthesized proteins, mutant proteins, newly damaged proteins and competing cytoplasmic lattice during cellular remodeling. These above mentioned protein machineries are collectively referred as “Clients”, which include cell surface receptor to various cytokines and huge array of protein molecules. In this scenario, pharmacological targeting of overloaded Hsp90, the molecular chaperone, causes simultaneous degradation of client proteins via an apparently understood mechanism and results in retardation of neoplastic progression [16–18]. Here we tried to unearth the mechanistic insight of Hsp90 inhibitor i.e. 17-AAG mediated neoplastic growth inhibition by modulating telomerase reverse transcriptase (TERT) and cyclin D1. Both, TERT and Cyclin D1 control cell cycle and cell proliferation of normal and neoplastic cells. Previously we reported that intraperitoneal administration of vincristine sulfate indirectly reduced TERT expression in experimentally induced sarcomatosis by arresting the cells at G2/M phase stage of cell cycle [9]. Previously it was also reported that 17-AAG inhibit telomerase assembly and activity *in vitro* in human JR8 melanoma cell line [19]. Our present study considered direct targeting of TERT *in vivo* by inhibiting the Hsp90 through 17-allylamino-17-demethoxygeldanamycin (17-AAG), which is a derivative of ansamycin family of anti-tumor antibiotic — geldanamycin. Currently 17-AAG is in Phase II clinical trial. In Phase-I trial, pharmacodynamic activity of the drug was evaluated by measuring Hsp70 activation and/or degradation of CDK4, a kinase, which phosphorylates Cyclin D1 [20–24]. In normal condition, CDK4 mediated phosphorylation of Cyclin D1 promotes cells to complete G1 phase of cell cycle and enter in S phase. We wanted to study the Cyclin D1 status after 17-AAG administration in the context of sarcomatosis related peritoneal ascites formation. Our study also addressed the peritoneal sarcomatosis related peripheral blood neutrophilia due to over expression of GM-CSF by peritoneal malignant ascitic sarcoma. We have followed up the possible modulation of GM-CSF production after 17-AAG administration in the sarcoma bearing mice as GM-CSF is one of the clients of Hsp90.

Ultimately, our present study tried to reduce chaperone overload in neoplastic condition like experimentally induced sarcomatosis related peritoneal ascites in mouse model by geldanamycin derivatives 17-AAG and hopefully it will bring some new light to those patients having aforementioned secondary neoplastic complicity.

MATERIAL AND METHODS

Animals. 10–12 weeks old Swiss albino mice (*Mus musculus*) weighing 20 to 24 g were selected from an inbred colony maintained under controlled room temperature (22±2°C) in the animal house of our institute. During the course of the study the animals

were fed on a diet consisting of 25.0% protein, 10.0% fiber, 5.0% fat, 9.0% minerals and access to water ad libitum, under standard conditions with a 12 h light dark period. Maximum six animals were housed in cage containing sterile paddy husk as bedding through out the experiment. The procedures followed were in agreement with the approved guide for the care and use of laboratory animals and Institutional Animal Ethical Committee (IAEC).

Sarcoma-180 transplantation. In this experiment, Crocker’s sarcoma (ascitic sarcoma-180) was taken as the source of malignant peritoneal ascites development. The ascitic fluid was drawn using an 18-gauge needle into sterile syringe. 100 µL of ascitic fluid was tested for microbial contamination, then viability was determined by erythrosin-B exclusion test and cells were counted using hemocytometer. The ascitic fluid was suitably diluted in sterile phosphate buffer saline to get a concentration of 3×10^6 cells/ml of ascitic suspension. This was injected intraperitoneally to obtain malignant ascitic effusion within 12 to 14 days.

Chemotherapy administration in vivo. We have used an Hsp90 inhibitor, 17-AAG in two divided doses (330 µg/kg b.w./day for first five days then next ten days with 166 µg/kg b.w./day) through intra-peritoneal route of inbred Swiss albino mice bearing full grown peritoneal malignant ascites of sarcoma-180 after twelve days of inoculation. The above drug administration schedule with fully developed tumor burden mice was considered for mimicking the late stage condition of malignant peritoneal ascites complicity in human being regarding any peritoneal or retroperitoneal sarcomatosis.

Peripheral blood hemogram study. We have randomly selected experimental and control animals from respective cages for blood hemogram profiling. Approximately 200 µL of blood was collected in heparinized vial by tail vein puncture from both the groups of animals. The hemoglobin concentration, total white blood cells (WBC), red blood cells, platelets, reticulocytes and differential WBC count were performed as per standard laboratory techniques.

Malignant ascitic cytology study. Malignant peritoneal ascitic fluid was drawn from both 1) control untreated malignant ascites bearing animals and 2) from 17-AAG treated malignant ascites bearing animals and washed by phosphate-buffered saline (PBS). Slides were prepared to study the cytology without damaging the cell membrane stained by Giemsa.

Erythrosin-B dye exclusion study. After 12–14 days of S-180 inoculation, 1.0 to 2.0 ml of malignant ascitic fluid was collected from control and 17-AAG treated animals and diluted in PBS to maintain a cell density of 1.0×10^6 /ml. The survival rate of tumor cells was evaluated by the Erythrosin-B (Sigma) dye exclusion technique using a hemocytometer (Rohem India) under an optical binocular light microscope (×400 magnification, Olympus).

Survival study. Chemotherapeutic efficacy can be accessed by median survival time (MST) and

increased life span (%ILs) of tumor bearing animals by the following formula.

$$\%ILS = \frac{\text{MST of the treated group} - \text{MST of the control group}}{\text{MST of the control group}} \times 100$$

Enhancement of life span (25% and above) of treated group over the control was considered as effective antitumor response. Additionally, we also studied growth pattern of the treated and untreated animals at regular interval which helped us to correlate the effect of 17-AAG on tumor burden of the animals.

Mitotic index study. Intraperitoneal treatment of 17-AAG might caused metaphase arrest of malignant peritoneal ascites in the peritoneum within 90 min of drug administration. Peritoneal ascitic fluid was collected by aspiration and washed with normal saline (0.9% NaCl) followed by further processing in hypotonic solution of 0.075M KCl at 37°C for about 15–25 min. Thereafter, cells were treated with acetic-methanol (1:3) fixative solution following vigorous vortexing to avoid the cell coagulation. The process was repeated for three times to increase the number of fixed cells. Slides were prepared through fixation by heat followed by Giemsa staining.

Mitotic indexing:

$$\text{Mitotic Index (MI)} = \frac{\text{TDC} \times 100}{\text{TC}}$$

(TDC= Total dividing cells, TC=Total Cell)

Cell cycle analysis. Flowcytometric cell cycle pattern was studied from 17-AAG treated and 17-AAG untreated control cell population by propidium iodide staining kit as per manufacturer instruction. Here, 20 000 events were analyzed by BD-FACS Calibur (Becton Dickenson, USA), using Cell Quest Pro software.

Flowcytometric analysis of malignancy related ascites for Hsp90, TERT, CyclinD1, PCNA and GM-CSF expression. In both the groups Hsp90, TERT, Cyclin D1, PCNA and GM-CSF expression pattern in peritoneal malignant ascites were studied by 15 minutes 1% Para-formaldehyde fixation and permeabilization by 90% ice-cold methanol followed by intracellular staining as described later. Five samples were incubated for 30 min with 2 μ L rabbit anti Hsp90 antibody (Cell Signaling Technology, Canada), rabbit anti TERT antibody (Santa Cruz Biotechnology, USA), Anti Cyclin D1-FITC tagged antibody (Abcam, UK), PCNA (Santa Cruz Biotechnology, USA), and Anti GM-CSF-FITC antibody (Biolegend, USA) respectively followed by addition of 2 μ L anti rabbit IgG-Alexafluor-488 (Invitrogen, USA) in untagged primary antibody coupled cells and incubated further in dark for 30–35 min. Excess fluorescence was then washed off with PBS. Samples were analyzed by BDFACS Calibur (Becton Dickenson, USA) using Cell Quest Pro software.

Statistics. All the value of hemogram, cell viability, mitotic index and FACS data were expressed as mean \pm SD (standard deviation). Statistical analysis was performed by Paired t Test ($2\alpha = 0.05$) and each experiment was performed three times.

RESULTS

Peripheral blood. Untreated sarcomatosis related ascites bearing animals showed leukemia like condition during peritoneal sarcoma burden condition and mostly sudden overshoot of hemoglobin, reticulocytes, RBC and WBC count. Mainly, in differential count, untreated animals showed an unusual elevation of neutrophils in comparison to lymphocyte population. In the normal Swiss albino strain of mouse, peripheral blood comprised of a higher proportion of lymphocyte (60–70%) over neutrophils (25–35%). On the other hand, treatment with 17-AAG (after 12 days of experimentally induced peritoneal sarcomatosis) showed a suppression of sudden neutrophilia in the differential count along with normal standards of total Hb, Reticulocyte, RBC and WBC count. All the results of comparative hemogram were verified by Student's 't' test and were statistically significant (Fig. 1 a, b, and Table 1).

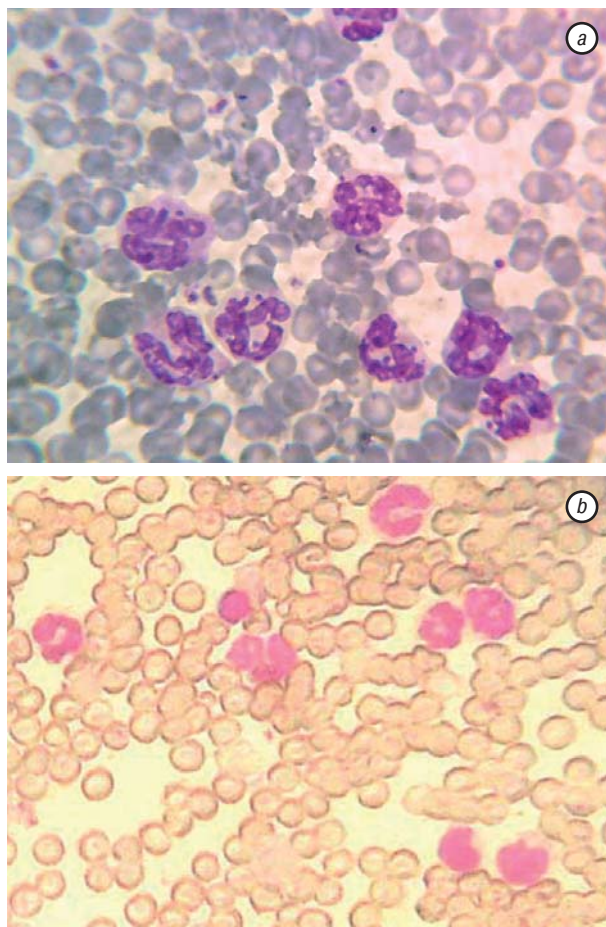


Fig. 1. (a) represented peripheral blood neutrophilia in the untreated group of animals with sarcomatosis related malignant ascites in peritoneum. Appearance of banded nucleated neutrophils in peripheral blood of untreated animals was the manifestation of malignant cell mediated GM-CSF over production. In contrary, (b) showed almost reversed hematological picture in the 17-AAG treated group of animals, which revealed a normal neutrophil count along with standard nuclear segmentation

Malignant ascitic cytology. Light microscopy of untreated and 17-AAG treated malignant peritoneal sarcomatosis related ascites showed the presence of usual large cells with high cytoplasmic to nuclear ratio. 17-AAG treated malignant cells showed cytoplas-

Table 1. Peripheral blood hemogram of untreated and 17-AAG treated groups of animals

Parameters	9 th days	15 th days	18 th days	24 th days	30 th days	45 th days	P value (Each experiment repeated for 3 times)
Hemoglobin (g/dl)							
Untreated S-180 animal	16.93±0.34	10.44±0.40	15.64± 0.6	**	**	**	P<0.0001
Treated S-180 animal	****	12.75±0.53	11.54±1.0	10.50±0.5	12.60±0.1	12.75±0.2	
Reticulocyte (%)							
Untreated S-180 animal	1.56±0.1	1.38±0.3	1.42± 0.34	**	**	**	P<0.0001
Treated S-180 animal	****	1.26±0.06	0.77± 0.1	1.95±0.3	1.92 0.15	1.68±0.13	
Total R Total RBC count (×10 ⁶ cell/ μL)							
Untreated S-180 animal	13.87±0.1	6.16±0.20	6.55±0.30	**	**	**	P<0.001
Treated S-180 animal	****	6.54±0.12	3.19±0.11	2.26±0.21	2.88±0.1	4.59±0.05	
Total WBC count (×10 ⁴ cell/μL)							
Untreated S-180 animal	7.8±0.5	6.7±0.13	6.7±0.20	**	**	**	P<0.0001
Treated S-180 animal	****	5.3±0.21	5.6±0.14	1.94± 0.3	3.96±0.23	4.11±0.5	
Differential count (%)							
Untreated							
Lymphocyte	10±1.3	11±2.1	15±1.24				P<0.0001
Neutrophils	87±1.5	86±2.3	84±1.5				
Eosinophils	0	0	0	**	**	**	
Basophiles	0	0	0				
Monocyte	3±0.2	3±1.1	1± 0.01				
Treated							
Lymphocyte		20±1.2	28±2.0	40±0.5	53±2.1	47±2.0	
Neutrophils		75±0.5	71±1.3	60±0.75	40±1.3	53±. 0.5	
Eosinophils	****	0	0	0	0	0	
Basophiles		0	0	0	0	0	
Monocyte		5±0.01	1±0.2	0	7±1.2	0	

Notes: ** Untreated malignant ascites bearing animals more or less survived up to 18 days from 1st day of passaging. **** We started the 17-AAG treatment after 12 days of tumor passaging.

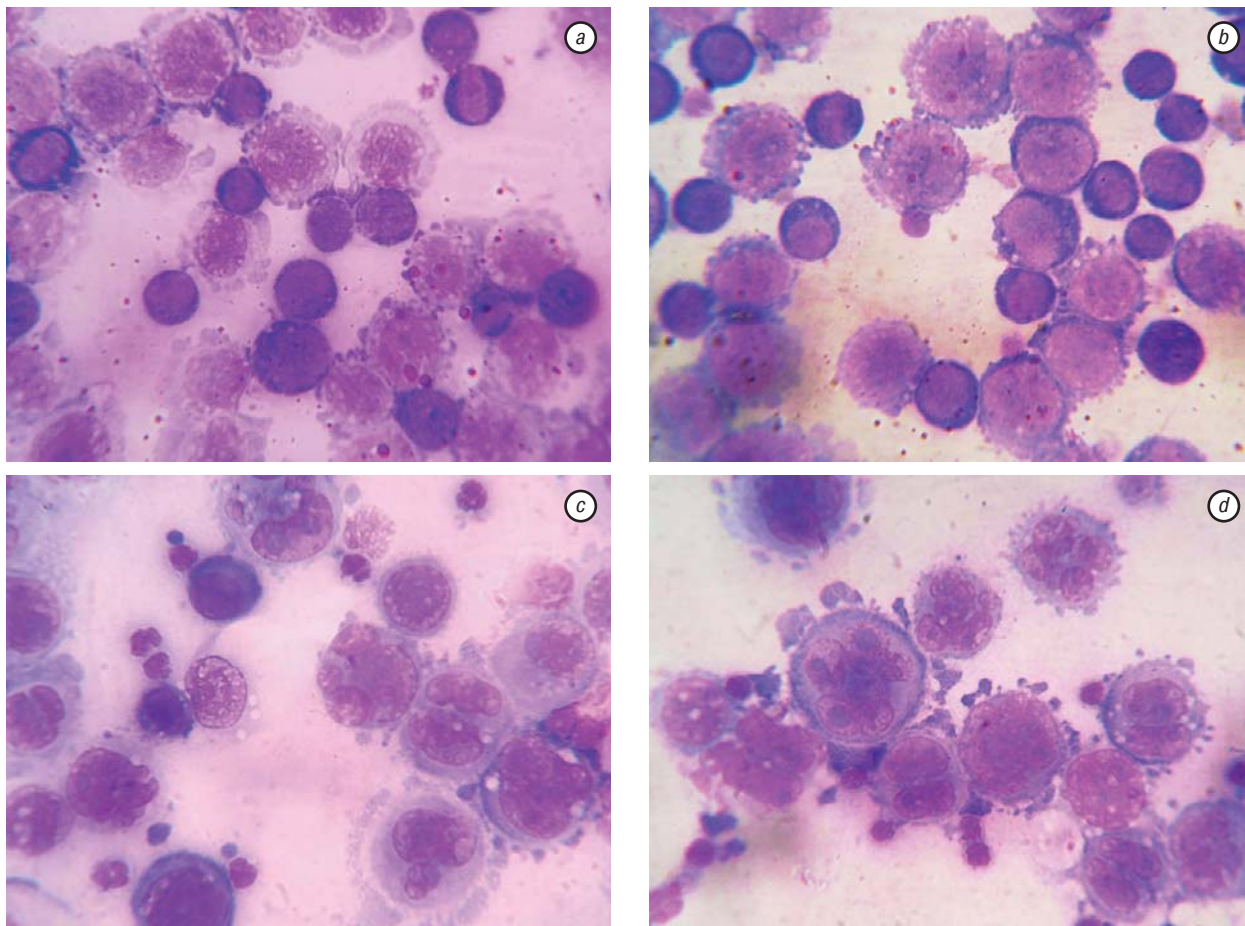


Fig. 2. (a) and (b) showed ascites cytology in the untreated group of animals. High cytoplasmic to nuclear ratio was evident from (a). Cytoplasmic basophilia was also documented in (b) with a few contaminating lymphocytes. (c) and (d) revealed the ascites cytology after 17-AAG treatment, which made malignant sarcoma cells error prone during the event of karyokinesis. Additionally, cytoplasmic to nuclear ratio was altered and as a result heterogeneous population of malignant cells appeared in the peritoneum of treated animals (c). Faulty karyokinesis was manifested by nuclear-cytoplasmic asynchrony and formation of micronucleus containing syncytial cells (d)

mic-nuclear asynchrony by producing syncytial cells. Additionally, a number of micronuclei were observed

inside the 17-AAG treated syncytial malignant cells. Cytoplasmic basophilia was also evident in untreated

malignant ascites in comparison to 17-AAG treated ascites. Furthermore, it was observed that geldanamycin treatment also deformed the uniformity of the plasma membrane of the experimental cells (Fig. 2 a–d).

Erythrosin-B dye exclusion study for cell viability. In comparison to the untreated animals (99.53%±0.6) the ascitic cell viability of the 17-AAG treated animals decreased to 43.71%±1.5 which was statistically significant ($P < 0.0001$) (Fig. 3) and correlated well with the efficacy of the 17-AAG treatment.

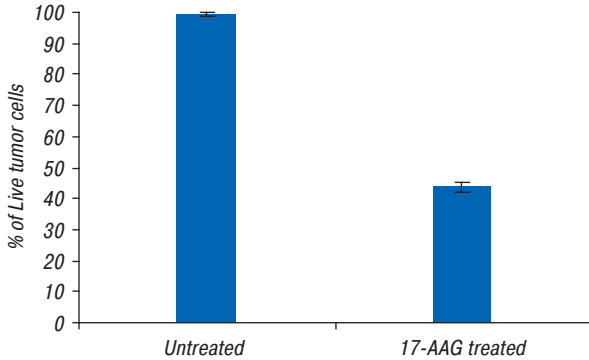


Fig. 3. Erythrosin-B dye exclusion test for cell viability assessment revealed that untreated malignant cells were more or less 100% viable in comparison to the 17-AAG treated group. On an average 44% cells were viable inside the peritoneum of the treated group of animals

Survival study. MST and %ILS varied significantly in both groups. MST value of untreated group was 10 days whereas the treated group MST showed 23 days. The survival range also improved from 3–11 days in untreated to 14–45 days in the treated group. Increased life span (%ILs) was observed in the 17-AAG treated group (130%) in comparison to the untreated group (Table 2).

Table 2. Effect of 17-AAG on MST and % increased life span (%ILS) of treated peritoneal sarcomatosis related ascites bearing mice

Groups	MST (Days) (Survival range)	% ILS
Untreated	10 (3–10)	
17-AAG treated	23 (14–45)	130

Mitotic index of untreated and 17-AAG treated peritoneal sarcoma. We designed the present experiment to evaluate the effect of 17-AAG on the tumor cell mitosis and chromosome equipartitioning mechanism during the event of metaphase. The untreated group showed 60.57% ± 1.91 MI in comparison to 17-AAG treated mice, which had 35.21 ± 1.30% MI (Fig. 4, a, b). Most of the cells of the treated group were found in the interphase stage of cell cycle in comparison to the untreated animals (Fig. 4, b).

Cell cycle analysis. Unlike 0.24% Go/G1 cells of untreated malignant ascites, 17AAG treated ascites showed 79.98% cells halted in G0/G1 phase. On the other hand high percentage of S phase cells (92.57%) was evidenced in untreated sample whereas 17AAG treatment reduced the number of S phase cells (18.24%) in treated group of animals. G2/M phase specific cells were 7.27% in case of untreated sample, whereas only 1.98% 17AAG treated cells were in G2/M phase stage (Fig. 5).

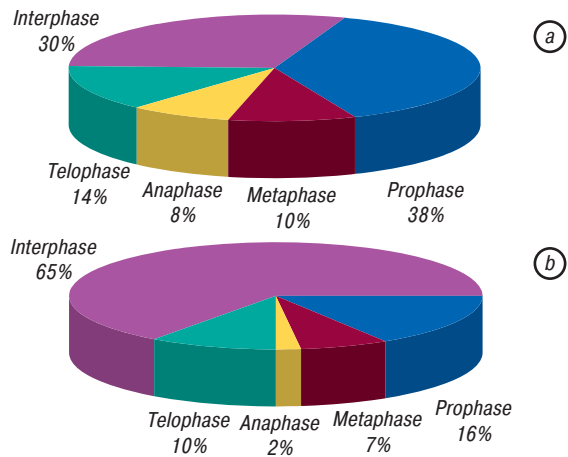


Fig. 4. Mitotic index (MI) of untreated group (a) represented 60.57% cells in mitosis and very negligible amount of cells were present in metaphase which demonstrated the rapid cell cycling nature of untreated malignant cells. Nearly 38% cells were in Interphase stage. (b) revealed the MI of 17-AAG treated group where 17-AAG treatment blocked the cells at Interphase (65%) and very low amount of cells were at metaphase (7%)

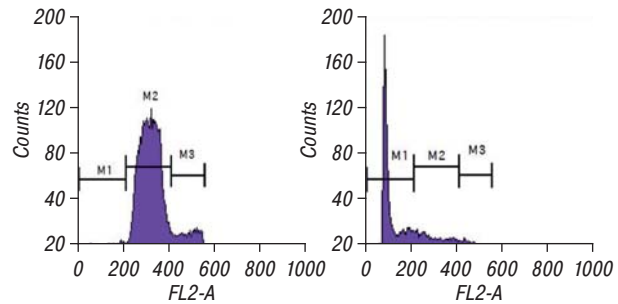


Fig. 5. Flowcytometric cell cycle study of untreated and 17AAG treated malignant ascites. M1, M2 and M3 are representative virtual marker of G0/G1, S and G2/M phases respectively

Flowcytometric analysis of Hsp 90 and its client proteins. Flowcytometric study of Hsp90, TERT, Cyclin D1, PCNA and GM-CSF expression pattern in peritoneal malignant sarcoma related ascites in the 17-AAG treated group showed downregulation of TERT (60.01 ± 1.22), cyclin D1(3.32 ± 0.24), PCNA(60.23 ± 0.02), GM-CSF (61.43 ± 0.4) and Hsp90 (209 ± 2.65) expression in comparison to the untreated group (TERT (172.90 ± 2.07), cyclin D1 (14.01± 0.2), PCNA (73.80 ± 0.1), GM-CSF (70.15 ± 1.2) and Hsp90 expression (248 ± 2.65)] (Fig. 6 and Table 3).

Table 3. Flowcytometrically determined mean fluorescence intensity (MFI) values of Hsp90 and its clients represented comparative expression pattern in untreated and treated groups of animals

Target proteins	MFI of untreated samples	MFI of treated samples	P value (Each experiment repeated for 3 times)
Hsp90	209 ± 2.65	46.32 ± 2.1	P<0.0001
TERT	172.90 ± 2.07	60.01 ± 1.22	P=0.0003
Cyclin D1	14.01± 0.2	3.32 ± 0.24	P=0.0001
PCNA	73.80 ± 0.1	60.23 ± 0.02	P<0.0001
GM-CSF	70.15 ± 1.2	61.43 ± 0.4	P<0.0001

DISCUSSION

In the present study we emphasized on the formation of peritoneal sarcomatosis (sarcoma-180) related malignant ascites and its associated pathophysiologies in syngenic adult Swiss albino mice. In our previous study we already showed that intraperitoneal vincristine administration reduced malignant ascite burden inside

the murine peritoneum by downregulating cellular senescence switch — TERT, a main regulatory component of the telomerase enzyme [9]. In corroboration with the previous study, here we again targeted TERT by a more efficient manner which was related to antagonizing Hsp90 by its newly developed pharmacological inhibitor-17-AAG. The Hsp90 inhibitor 17-AAG is the derivative of ansamycin and it competes with cellular ATP to bind at NH₂-terminal ATP/ADP binding domain of Hsp90 to block its chaperone functions, leading to destabilization and proteosomal degradation of the client proteins (e.g. TERT, cyclin D1 etc). Deregulation of the client's folding procedure by Hsp90 brought a number of direct and indirect consequences inside the cells. Our present work also revealed the changes step by step in the event like sarcoma related malignant ascites development inside the murine peritoneum.

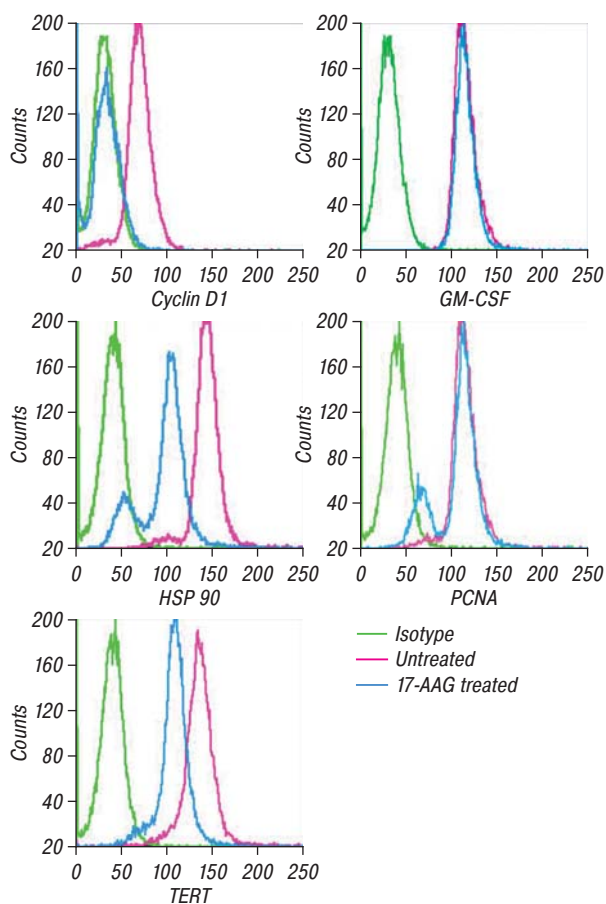


Fig. 6. Comparative flowcytometric analysis of Hsp90 and its client proteins in the untreated and 17-AAG treated groups of animals

In the untreated group we observed abnormal peripheral blood hemogram with a huge overshoot of neutrophil population in comparison to lymphocytes. Furthermore this peripheral blood neutrophilia was steadily present and often, in some experimental animals, lymphocytes and other granulocytes were totally replaced by aforementioned neutrophilia in peripheral blood during differential count. The most possible explanation of the persisting peripheral neutrophilia in ascitic sarcoma bearing mice was due to the constant over stimulation of resident bone marrow neutrophil population by endogenous GM-CSF which was not found in normal condition. The

overshoot of GM-CSF production as evidenced by FACS analysis furthered validated the reason of neutrophilia in untreated group. The immature marrow neutrophil pool (mostly banded neutrophils) shifted from endosteal niche to vascular niche to peripheral blood due to higher expression of GM-CSF. The reason behind the over expression of GM-CSF by sarcoma cells is still obscure. Constitutive expression of any protein is actually the result of mutation and these mutated proteins usually crowd the cellular environment rapidly. Ramification of the unfolded mutated protein is generally managed by over expressed Hsp90 in response to oncogenic stress. So, in the present study we targeted the Hsp90 by 17-AAG, resulted in deregulation in refolding process of GM-CSF by competitive binding with Hsp90 and produced faulty unstable non-functional proteins (GM-CSF) in cellular environment and consequently stopped the procedure of over stimulation of marrow neutrophil storage. Ultimately the beneficial effect of 17-AAG was documented in the post treatment hemogram of the treated experimental mice.

We observed micronucleus formation and nuclear-cytoplasmic asynchrony in the treated group in comparison to the untreated group. Nuclear-cytoplasmic asynchrony with more than two nucleoli was due to the deregulation at cytokinesis and polyploidy induction in malignant cells. But, multinucleated cells with presence of one or two or three micronuclei and one large nucleus were the manifestation of improper assortment of chromosome during metaphase stage. It evidenced deregulation of Hsp90 and cytoskeletal protein interaction during metaphase in presence of Hsp90 inhibitor like 17-AAG. Interestingly no significant amounts of malignant cells were found at the metaphase stage during the mitosis study and mitotic indexing. Our previous study related to vincristine revealed that the vinca alkaloid reduced the mitotic index of malignant cells by metaphase blocking mechanism [9]. The present study established the decrease of MI of the malignant cells without blocking at metaphase stage by 17-AAG treatment. However, in comparison to the untreated group, treated group showed higher percentage of cells at interphase stage which was furthered explained by flowcytometric investigation of cell cycle specific marker protein expression.

We have considered the status of a few important cell cycle specific proteins by flow cytometry e.g. cyclinD1, PCNA and TERT to clarify the increase of interphase cells. Cyclin D1 was used as G0/G1 phase marker. PCNA was used for S-phase specific marker where DNA replication takes place and the TERT was used as the late S phase to early G2 phase entry marker which causes cellular immortality. The 17-AAG treated group showed drastic downregulation of Cyclin D1 in comparison to the untreated group which signified the direct competition of Cyclin D1 with 17-AAG for the N-terminal of Hsp90 and the remnant unfolded Cyclin D1 degraded soon. As a result of Cyclin D1 degeneration, significant amount of cells were blocked at G0/G1 stage which might be the possible reason for the increased interphase cell count during MI.

A moderate depression of PCNA was observed in the treated group in comparison to the untreated group. 17-AAG treatment hampered PCNA expression and stability during the DNA replication. Downregulated PCNA in 17-AAG treated group also signified that very few numbers of cells with active DNA replication procedure were present in S phase. The TERT expression data also added an extra dimension in the context of cell cycling status of the treated group. Unlike untreated group, treated group showed a sharp down-regulation of TERT expression, which manifested two things: 1) 17-AAG efficiently blocked Hsp90 guided client processing mechanism for which cancer cell proliferation and self renewed process was under limitation as both were being maintained by TERT. 2) Low viability of treated peritoneal sarcoma cells due to improper DNA end replication.

In conclusion, we can say that 17-AAG treatment mediated controlling of a number of oncoprotein expression along with Hsp90 itself increase the life expectancy of the treated animals group in comparison to untreated one. Targeting Hsp90 by 17-AAG also caused overcrowding of misfolded protein in malignant cells which ultimately reduced tumor burden in mice by diminished tumor cell viability and flowcytometric TERT expression study would be considered as a novel diagnostic and prognostic tool for sarcoma progression.

CONFLICT OF INTEREST

All authors unanimously declared no potential conflict of interest.

ACKNOWLEDGEMENT

We are thankful to the Director of the Calcutta School of Tropical Medicine.

REFERENCES

- Oei T N, Jagannathan J P, Ramaiya N, Ros P R. Peritoneal sarcomatosis versus peritoneal carcinomatosis: imaging findings at MDCT. *Am J Roentgenol* 2010; **195**: W229–35.
- Bilici A, Uygun K, Altun E, *et al.* Primary peritoneal spindle cell sarcoma presented with massive ascites: a case report. *J BUON* 2009; **14**: 721–3.
- Yokoi M, Hosokawa K, Funaki H, *et al.* A case of retroperitoneal dedifferentiated liposarcoma successfully treated with IFM and CDDP. *Gan To Kagaku Ryoho* 2009; **36**: 2114–6.
- Colizza S, Di Maurizio P, Bracci F, *et al.* Diffuse peritoneal liposarcoma — report of one case. *J Surg Oncol* 1981; **18**: 147–51.
- Leal R, Lewin M, Ahmad I, Korula J. Peritoneal Kaposi's sarcoma: a cause of ascites in acquired immunodeficiency syndrome. *Dig Dis Sci* 1994; **39**: 206–8.
- Bairy KL, Satinath S, Jagetia GC, *et al.* Evaluation of intraperitoneal vincristine in malignant peritoneal effusion. *Ind J Physiol Pharmacol* 2003; **47**: 270–8.
- Runowicz C. Should patients with ovarian cancer receive intraperitoneal chemotherapy following initial cytoreductive surgery? *Nat Clin Pract Oncol* 2006; **3**: 416–7.
- Armstrong DK, Bundy B, Wenzel L, *et al.* Intraperitoneal cisplatin and paclitaxel in ovarian cancer. *N Engl J Med* 2006; **354**: 34–43.
- Chaklader M, Das P, Pereira JA, *et al.* Telomerase reverse transcriptase (TERT) expression and role of vincristine sulfate in mouse model of malignancy related peritoneal ascites: an experimental metastatic condition. *Exp Oncol* 2011; **33**: 83–9.
- Csermely P. Proteins, RNAs and chaperones in enzyme evolution: a folding perspective. *Trends Biochem Sci* 1997; **22**: 147–9.
- Hartl FU. Molecular chaperones in cellular protein folding. *Nature* 1996; **381**: 571–9.
- Thirumalai D, Lorimer GH. Chaperonin-mediated protein folding. *Ann Rev Biophys Biomol Struct* 2001; **30**: 245–69.
- Schneider C, Sepp-Lorenzino L, Nimmegern E, *et al.* Pharmacologic shifting of a balance between protein refolding and degradation mediated by Hsp90. *Proc Natl Acad Sci USA* 1996; **93**: 14536–41.
- Czar MJ, Welsh MJ, Pratt WB. Immunofluorescence localization of the 90-kDa heat-shock protein to the cytoskeleton. *Eur J Cell Biol* 1996; **70**: 332–0.
- Redmond T, Sanchez ER, Bresnick EH, *et al.* Immunofluorescence colocalization of the 90 kDa heat-shock protein and microtubules in interphase and mitotic mammalian cells. *Eur J Cell Biol* 1989; **50**: 66–75.
- Bagatell R, Whitesell L. Altered Hsp90 function in cancer: a unique therapeutic opportunity. *Mol Cancer Ther* 2004; **3**: 1021–30.
- Becker B, Multhoff G, Farkas B, *et al.* Induction of Hsp90 protein expression in malignant melanomas and melanoma metastases. *Exp Dermatol* 2004; **13**: 27–32.
- Dias S, Shmelkov SV, Lam G, Rafi S. VEGF(165) promotes survival of leukemic cells by Hsp90-mediated induction of Bcl-2 expression and apoptosis inhibition. *Blood* 2002; **99**: 2532–40.
- Villa R, Folini M, Porta CD, *et al.* Inhibition of telomerase activity by geldanamycin and 17-allylamino, 17-demethoxygeldanamycin in human melanoma cells. *Carcinogenesis* 2003; **24**: 851–9.
- Zhang H, Burrows F. Targeting multiple signal transduction pathways through inhibition of Hsp90. *J Mol Med* 2004; **82**: 488–99.
- DeBoer C, Meulman PA, Wnuk RJ, Peterson DH. Geldanamycin, a new antibiotic. *J Antibiot (Tokyo)* 1970; **23**: 442–7.
- Supko JG, Hickman RL, Grever MR, Malspeis L. Pre-clinical pharmacologic evaluation of geldanamycin as an anti-tumor agent. *Cancer Chemother Pharmacol* 1995; **36**: 305–15.
- Schulte TW, Neckers LM. The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin binds to HSP90 and shares important biologic activities with geldanamycin. *Cancer Chemother Pharmacol* 1998; **42**: 273–9.
- Yano M, Naito Z, Yokoyama M, *et al.* Expression of hsp90 and Cyclin D1 in human breast cancer. *Cancer Lett* 1999; **137**: 45–51.