Biochimica et Biophysica Acta 1840 (2014) 294–302

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Tumor growth retardation and chemosensitizing action of fatty acid synthase inhibitor orlistat on T cell lymphoma: Implication of reconstituted tumor microenvironment and multidrug resistance phenotype

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ARTICLE INFO

Article history:
Received 16 May 2013
Received in revised form 23 August 2013
Accepted 13 September 2013
Available online 20 September 2013

Keywords:
Apoptosis
Chemosensitization
Orlistat
T cell lymphoma
Fatty acid synthase

ABSTRACT

Background: Orlistat, a fatty acid synthase (FASN) inhibitor, has been demonstrated to inhibit tumor cell survival. However, the mechanism(s) of its tumor growth retarding action against malignancies of hematological origin remains unclear. It is also not understood if the antitumor action of orlistat implicates modulated susceptibility of tumor cell to anticancer drugs. Therefore, the present investigation focuses to study the antitumor and chemosensitizing action of orlistat in a murine host bearing a progressively growing T cell lymphoma.
Methods: Tumor-bearing mice were administered with vehicle alone or containing orlistat followed by administration of PBS with or without cisplatin. Tumor progression and survival of tumor-bearing host were monitored along with analysis of tumor cell survival and apoptosis. Tumor ascitic fluid was examined for pH, NO and cytokines. Expression of genes and proteins was investigated by RT-PCR and western blot respectively. ROS was analyzed by DCFDA staining and FASN activity by spectrophotometry.
Results: Orlistat administration to tumor-bearing mice resulted in tumor growth retardation, prolonged life span, declined tumor cell survival and chemosensitization to cisplatin. It was accompanied by increased osmotic fragility, modulated acidosis, expression of ROS, NO, cytokines, MCT-1 and VH^+ ATPase, Bcl2, Caspase-3, P53, inhibited FASN activity and declined expression of MDR and MRP-1 proteins.
Conclusion: Orlistat manifests antitumor and chemosensitizing action implicating modulated regulation of cell survival, reconstituted-tumor microenvironment and altered MDR phenotype.
General significance: These observations indicate that orlistat could be utilized as an adjunct regimen for improving antitumor efficacy of cisplatin.

1. Introduction

Malignant cells are bestowed with a robust proliferative ability associated with vigorous membrane biogenesis, adaptability and mammoth cell survival instinct, rendering them capable of generating chemoresistance, leading to development of therapeutic approaches for targeting one or more such physiological traits of tumor cells [1–6]. Tumor cell depends on de novo fatty acid synthesis for their membrane biogenesis, which is catalyzed by fatty acid synthase (FASN) [6–11]. FASN is a multimeric enzyme, which catalyzes synthesis of palmitate from malonyl- and acetyl-CoA in the presence of NADPH [12]. Thus one of the emerging novel anticancer approaches implicates inhibition of FASN catalyzed lipid synthesis, employing chemicals and siRNAs [6,7,9,10]. Therefore, FASN inhibitors like orlistat, cerulenin, and c75 are being tested for their antitumor potential [6–9,13,14]. Reports indicate that orlistat, which binds to the thioesterase domain of FASN [6,9,10], manifests its cytotoxic action against a wide spectrum of malignant cells [13–15]. Nevertheless, orlistat does not induce carnitine palmitoyl transferase (CPT) compared to cerulenin and c75 and is therefore, considered safer [14,16]. Although the mechanism(s) of the antitumor action of orlistat is mainly reported to depend on events triggered by FASN inhibition, the associated downstream molecular events need to be worked out further in a tumor-specific manner. Considering the fact that only sporadic reports are available with regard to antitumor action and underlying mechanism(s) of FASN against malignancies of hematological origin, in a previous study based on in vitro analysis, we reported some of the molecular mechanism(s) associated with the cytotoxic action of orlistat against a murine T cell lymphoma. However, it remains unclear if such in vitro cytotoxic potential of orlistat could have translational application to retard tumor progression in vivo. Consequently, it will also be imperative to work out the additional novel mechanism(s),

Abbreviations: DCFDA, dichlorodihydrofluorescein diacetate; DL, Dalton's lymphoma; FASN, fatty acid synthase; iNOS, inducible nitric oxide synthase; MRP-1, multidrug resistant associated protein-1; MDR, multidrug resistant protein; NO, nitric oxide; ROS, reactive oxygen species

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http://dx.doi.org/10.1016/j.bbagen.2013.09.020

operational exclusively under in vivo conditions, mediating the antitumor action of orlistat.

Accumulating experimental evidences indicate the indispensible role of acidosis in promoting tumor growth, which in turn is regulated by unique repertoire of membrane-associated pH regulators [3,17–19]. Since the main mode of the antitumor action of orlistat is mediated by inhibition of membrane biogenesis, it is likely that it may alter the expression of pH regulators on tumor cell membrane, which could also constitute one of the mechanisms associated with the antitumor action of orlistat in vivo depending on altered pH regulation. Moreover, it remains to be investigated if administration of orlistat to tumor-bearing hosts can also be utilized as an adjunct therapeutic strategy to modulate the antitumor efficacy of other chemotherapeutic regimens by rendering the tumor cells, with inhibited membrane biosynthesis and deregulated pH control, vulnerable to their cytotoxicity.

In view of the aforementioned lacunae, the present investigation was undertaken to examine tumor growth retarding potential and underlying mechanism(s) of orlistat against a progressively growing tumor in a murine tumor model of transplantable T cell lymphoma, designated as Dalton's lymphoma [20–22]. The study demonstrates that orlistat can be used as adjunct therapeutic regimen to potentiate antitumor action of cisplatin associated with a reconstituted tumor microenvironment and reversal of MDR phenotype.

2. Materials and methods

2.1. Mice and tumor system

Pathogen-free inbred adult mice of BALB/c (H-2^d) strain were used at 8–12 weeks of age. The mice were procured from the animal house facility of the Banaras Hindu University approved by the institutional animal ethical committee and kept in the animal rooms of the School of Biotechnology. The mice received food and water ad libitum and were treated with utmost humane care. Tumor (Dalton's lymphoma) is maintained in ascitic form by serial transplantation in BALB/c mice or in an in vitro cell culture system by serial passage. Irrespective of whether the tumor cells were obtained from the in vitro culture system maintained as suspension cultures or from the ascitic fluid they exhibited similar phenotypic features. Serial passage of tumor cells in mice was carried out by transplanting 1×10^5 tumor cells $mouse^{-1}$ in 0.5 ml phosphate buffered saline (PBS).

2.2. Reagents

All reagents used were of tissue culture and/or analytical grade. Tissue culture medium RPMI 1640 was purchased from Hyclone (USA), supplemented with 20 μg/ml gentamycin, 100 μg/ml streptomycin, 100 IU penicillin purchased from Himedia (India) and 10% fetal calf serum from Hyclone (USA), henceforth, referred to as complete medium. Orlistat (USV Ltd., India) was purchased locally. Dichlorodihydrofluorescein diacetate (DCFDA), acetyl-CoA and malonyl-CoA were purchased from Sigma-Aldrich (USA). NADPH was purchased from SRL Pvt. Ltd. (India). Antibodies against the indicated proteins were obtained from Imgenex (USA), BD Pharmingen (USA), Sigma-Aldrich (USA), Santa Cruz Biotechnology, GeneTex (USA) and Chemicon (UK). Secondary antibodies conjugated to alkaline phosphatase were obtained from Bangalore Genie (India). Primers for RT-PCR were purchased from Abion International AG (Germany) and Integrated DNA Technologies (USA). BCIP/NBT was purchased from Amresco (USA).

2.3. Protocol for orlistat administration to tumor-bearing mice

Mice in a group of 9 each were used for examining the effect of orlistat on the indicated parameters. Orlistat was solubilized and administered following a protocol described in Fig. 1. Tumor cells (1×10^5cells) were transplanted to mice followed by intraperitoneal (*i.p.*) administration of vehicle alone (control) or containing of orlistat 48 h after tumor transplantation in a dose of 240 mg/kg/mice/day [23], till day 14 post tumor transplantation. Both control and experimental mice were then administered 0.5 ml PBS with or without cisplatin (5 mg/kg) on day 14 post tumor transplantation. Tumor cells and tumor ascitic fluid were harvested on day 16 post tumor transplantation.

2.4. Cell survival assay

Cell survival was determined by standard MTT assay according to a method described earlier [24] with slight modifications. MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] (5 mg/ml in PBS) was added to each well (50 μl/well) of the culture plate containing 200 ml medium and incubated at 37 °C for 4 h. The medium was then carefully removed, without disturbing the dark blue formazan crystals. Fifty microliter DMSO was added to each well and mixed thoroughly to dissolve the formazan crystals. Plates were then read on a microplate reader (Labsystems, Finland) at a wavelength of 540 nm. Readings are presented as % survival relative to control.

2.5. Annexin V staining for enumerating apoptotic cell population

Tumor cells were stained with annexin-V FITC as described earlier [24]. Tumor cells were washed thrice with PBS and resuspended in binding buffer containing annexin-V. After incubation at room temperature for 20 min, the cells were analyzed by a fluorescence microscope (Nikon, Japan).

2.6. ELISA for detection of cytokines

A standard ELISA was performed to detect the presence of the indicated cytokines in ascitic fluid of control and orlistat-administered tumor-bearing mice following a method described earlier [14]. Briefly, polystyrene microwell plates (Tarsons, Kolkata, India) were coated

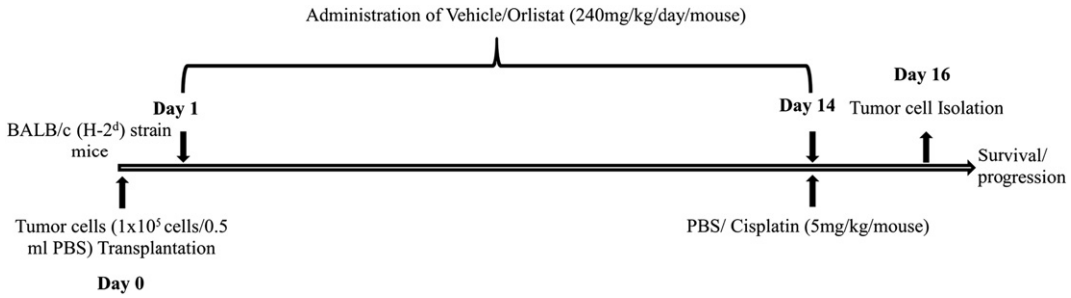

Fig. 1. Protocol for in vivo administration of orlistat and cisplatin to tumor-bearing mice. Mice were transplanted with tumor cells (1×10^5 cells/0.5 ml PBS) followed by administration of vehicle alone or containing orlistat (240 mg/kg/day) up to day 14 post tumor transplantation. On day 14, mice were administered with PBS alone or containing cisplatin (5 mg/kg). Tumor progression was monitored by measuring change in body weight and survival of tumor-bearing mice. Tumor cells were harvested on day 16 post-tumor transplantation for monitoring the indicated parameters.

with 10 μg of protein sample and incubated overnight at 4 °C. In the negative control, test samples were not added to wells of ELISA plates and were processed for subsequent steps in the same way as described for the experimental sets. The plates were washed with 0.15 M PBS containing 0.1% (v/v) Tween 20 (PBS-Tween). Unbound sites were saturated with PBS containing 1% bovine serum albumin (BSA). The plates were again washed with PBS-Tween followed by addition of antibodies against the indicated proteins at a dilution of 1:1000. The plates were incubated at 37 °C for 60 min followed by addition of 50 μl of p-nitrophenyl phosphate (NPP) (1 mg/ml) in enzyme substrate buffer. The absorbance was measured after 10 min at 405 nm in an ELISA plate reader (Labsystems, Finland) and the concentration of cytokines is presented as pg/ml.

2.7. Western immunoblot analysis

Western immunoblot analysis for detection of indicated proteins was carried out following a method described earlier [22]. Cells were washed with chilled PBS and lysed in 50 μl of lysis buffer (20 mM Tris–Cl, pH 8.0, 137 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 2 mM EDTA; 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin containing aprotinin (0.15 U ml^{-1})) for 20 min at 4 °C. Protein content in each sample was determined by using standard Bradford method. Twenty micrograms of Triton X-100 solubilized proteins was separated on 10% SDS-polyacrylamide gel at 20 mA. The gel was processed further for immunoblotting. The separated proteins were transferred onto a nitrocellulose membrane (Sartorius, Germany) (1.5 h at 150 mA), immunoblotted with antibodies against indicated proteins and probed with a secondary antibody: anti-rabbit IgG conjugated to alkaline phosphatase and detected by a BCIP/NBT solution (Amresco, USA). Equal loading of proteins was determined by using equal cell number for preparation of lysates, loading of equal protein content and immunoblotting of β-actin.

2.8. RT-PCR for expression of iNOS and FASN

RT-PCR analysis for the expression of mRNA of indicated genes was carried out according to a method described earlier [25] using a one step RT-PCR cell to cDNA kit (Ambion, USA). Primer sequences for various genes are shown in Table 1. PCR was performed for 15 min to make cDNA at 50 °C. The amplification was carried out for 30 cycles with initial denaturation at 94 °C for 2 min followed by annealing (annealing temperature as per respective primer design) for 30 s and elongation at 72 °C for 30 s. The samples were separated on an agarose gel (1%) containing ethidium bromide (0.3 μg/ml). Bands were visualized and analyzed on a UV-transilluminator (Bio-Rad, Australia).

2.9. Assay of FASN activity

The FASN activity was assayed in cell-free culture supernatant following a spectrophotometric method described by Ross et al. [15] with slight modifications. Briefly, tumor cells (1×10^6 cells/ml) of control and experimental groups were lysed in a lysis buffer (1 mM EDTA, 150 mM NaCl, 100 μg/ml phenylmethylsulfonyl fluoride, and 50 mM Tris–HCl pH 7.3) by vortexing followed by sonication. The cell lysates were centrifuged (16,000 ×*g*, 15 min) and protein content in the supernatant was determined by using the standard Bradford method. The supernatant was mixed in a reaction mixture containing potassium phosphate buffer 200 mM (pH 7.0), 1 mM EDTA, 1 mM dithiothreitol, 30 μM acetyl-CoA, and 0.24 mM NADPH, to a final volume of 300 μl. Absorbance of the reaction mixture was then measured by a spectrophotometer (Jasco, USA) at 340 nm for 3 min to measure NADPH oxidation. Malonyl-CoA (50 μM) was then added to the reaction mixture and absorbance was measured for another 10 min to determine FASN-dependent oxidation of NADPH. Rates were corrected for the background rate of NADPH oxidation in the presence of acetyl-CoA. FASN activity is presented as nmol NADPH oxidized/min/mg protein.

Table 1
Primer sequences for RT-PCR analysis.

Gene	Primer sequence
FASN	F-5′-AGGGGTCGACCTGGTCCTCA-3′
	R-5′-GCCATGCCCAGAGGGTGGTT-3′
iNOS	F-5′-ACGTGCGTTACTCCACCAACAA-3′
	R-5′-CATAGCGGATGAGCTGAGCATT-3′
β-Actin	F-5′-GGCACAGTGTGGGTGAC-3′
	R-5′-CTGGCACCACACCTTCTAC-3′

2.10. Measurement of intracellular reactive oxygen species (ROS)

ROS measurement was carried out as described by Furuta et al. [26] with slight modifications. Tumor cells obtained from control and experimental groups were washed followed by incubation with HBSS containing the fluorescent dye DCFDA at a final concentration 0.1 mM. The cells were further incubated at 37 °C for 45 min, followed by washing with PBS. Cells stained with the dye were visualized under a fluorescence microscope (Nikon, Japan) at a magnification of 400× and photographed. The amount of staining was quantified by MCID software.

2.11. Nitrite assay

The concentration of stable nitrite NO_2^-, the end product from NO generation, was determined by the method described earlier on the Griess reaction [22]. Test samples were incubated with an equal volume of Griess reagent [1 part of 1% (w/v) sulfanilamide in 2.5% H_3PO_4 plus 1 part of 0.1% (w/v) naphthyl ethylene diaminedihydrochloride; two parts being mixed together within 12 h of use and kept chilled] at room temperature for 10 min in a 96-well microtiter plate. The absorbance at 540 nm was determined with an automatic ELISA plate reader (Labsystems, Finland). Nitrite content was quantified by extrapolation from a standard curve of $NaNO_2$ in each experiment. In all the experiments nitrite content in the wells containing medium without cells was also measured and subtracted.

2.12. Osmotic fragility test

Osmotic fragility test was performed as described earlier [27]. Tumor cells (1×10^6 cell/ml) harvested from control and experimental tumor-bearing mice were incubated in saline with indicated concentration of NaCl for 10 min at 37 °C. Saline solution was then replaced with complete medium and cells were further incubated for 10 min at 37 °C. Viable cell count was performed using standard trypan blue dye exclusion test.

2.13. Statistical analysis

All experiments were conducted thrice in triplicate. The statistical significance of differences between test groups was analyzed by Student's *t* test. The difference was considered significant when p was less than 0.05.

3. Results

3.1. Tumor growth retarding and chemosensitizing action of orlistat

We checked the effect of orlistat-administration to tumor-bearing host on tumor progression and chemosensitization to cisplatin. Tumor-bearing mice were administered with vehicle alone (control) or containing orlistat (240 mg/kg) followed by administration of cisplatin (5 mg/kg) as per protocol described in the Materials and methods (Fig. 1). Tumor progression was monitored by measuring change of body weight and survival of tumor-bearing mice. Results are shown in Fig. 2a,b. Administration of orlistat or cisplatin alone

to tumor-bearing mice resulted in retardation of tumor progression compared to control. However, maximal effect on tumor growth retardation was observed in tumor-bearing mice administered with cisplatin following pre-exposure to orlistat (Fig. 2c,d,e). The volume of tumor ascitic fluid and tumor cell density significantly declined in tumor-bearing hosts administered with orlistat compared to control. Maximum inhibitory effect was, however, observed upon administration of cisplatin to orlistat-administered mice. In order to understand the mechanism of declined tumor load, tumor cells harvested from each test group were analyzed for survival by MTT assay and apoptotic cell population by annexin-V staining (Fig. 2f,g). Cell survival was observed to significantly decrease whereas the apoptotic cell population increases in tumor-bearing mice administered with orlistat, which was further, augmented in an additive manner in orlistat-plus-cisplatin administered groups.

3.2. Antitumor and chemo-sensitizing action of orlistat is associated with modulated expression of cell survival regulatory molecules

Since we observed that the tumor growth retarding and chemo-sensitizing action of orlistat implicated a decline in tumor cell number associated with augmented induction of apoptosis, in the next experiment we examined if the same was also associated with altered expression of some key cell survival regulatory molecules. Lysate of tumor cells (1×10^6 cell/ml) harvested from control and experimental groups was analyzed by western blotting for expression of indicated apoptosis regulatory proteins (Fig. 3a). The expression of caspase-3 and p53 was elevated in tumor cell harvested from orlistat-administered group, which was further up-regulated in the group administered with orlistat followed by cisplatin. On the other hand the expression of Bcl2 declined in orlistat-treated tumor-bearing mice with further inhibition upon administration of cisplatin. Since the expression pattern of aforesaid proteins and other cell survival regulatory molecules is controlled upstream by cytokines, we also checked the level of IL-6, IL-10 and IFN-γ in tumor ascitic fluid (Fig. 3b). The level of IL-6 and IFN-γ showed a significant increase in the ascitic fluid of tumor-bearing mice administered with orlistat, which was further augmented upon cisplatin administration. However, the level of IL-10 in the ascitic fluid declined in orlistat and orlistat plus cisplatin groups.

Since reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS) also play a crucial role in regulation of tumor cell survival, we also examined the effect of orlistat administration with or without cisplatin on the level of NO in tumor ascitic fluid (Fig. 3c) along with analysis of the expression of its regulating gene iNOS (Fig. 3d) and ROS (Fig. 3e). The level of NO was found to increase in ascitic fluid of orlistat-treated group, which was further elevated following cisplatin administration. The expression pattern of iNOS showed a similar trend with maximum up-regulation in orlistat and orlistat plus cisplatin-administered groups. Similarly the expression of ROS was found to be significantly elevated in tumor cells of orlistat-administered group with further augmentation upon cisplatin administration.

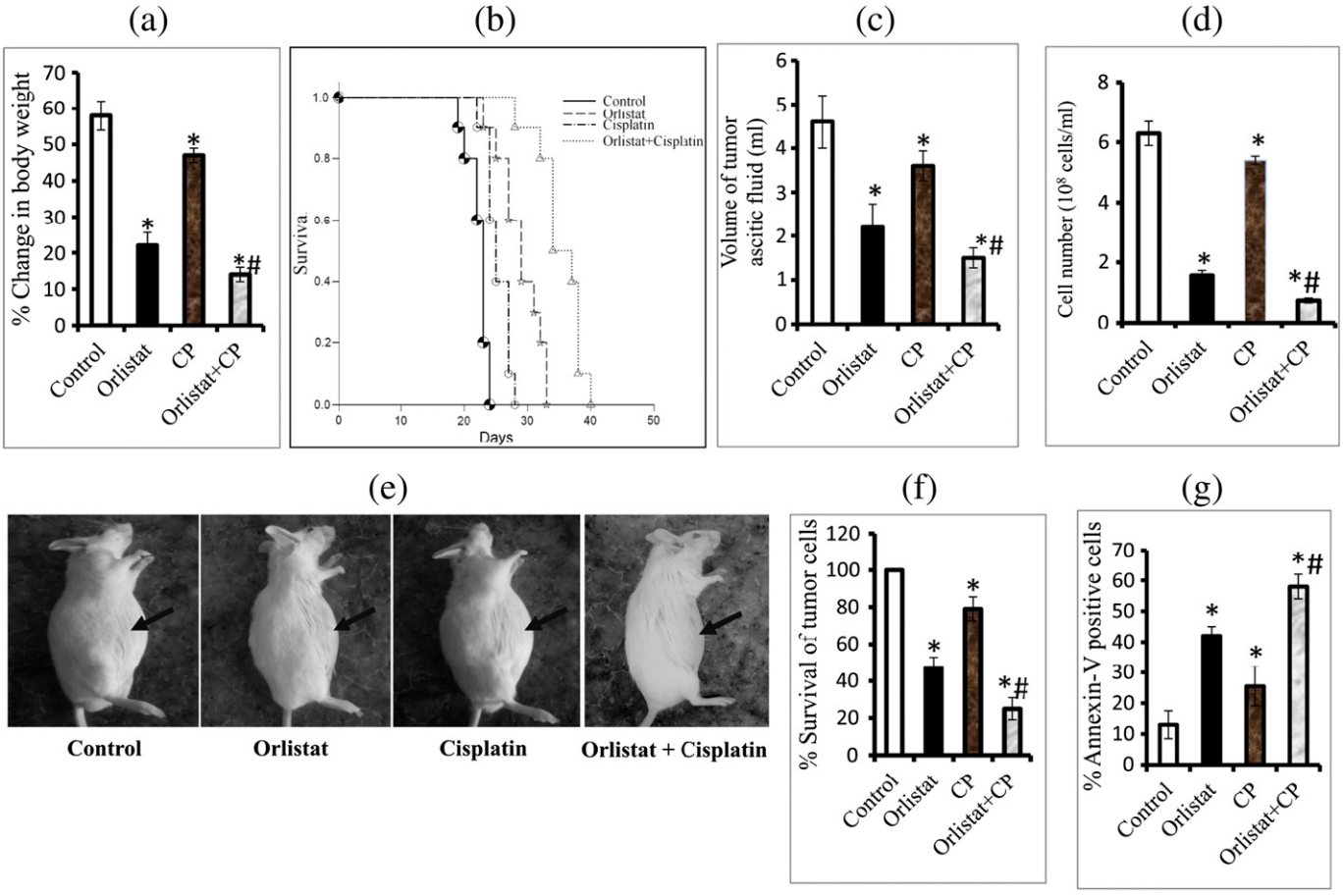

Fig. 2. Orlistat retards tumor progression and primes antitumor activity of cisplatin. Orlistat and cisplatin were administered to tumor-bearing mice as described in the Materials and methods. Tumor progression was monitored by measuring change of body weight calculated on day 20 post-tumor transplantation (a), survival of tumor-bearing mice (b), volume of tumor ascitic fluid (c) and enumeration of viable tumor cells (d) with monitoring of body contour as external anatomical indicator of tumor size (e). Tumor cell survival (f) and induction of apoptosis (g) were also estimated in tumor cells harvested from control and indicated experimental groups. Values shown in a, c, d, f and g are mean ± SD of three independent experiments done in triplicate. $^{*}p < 0.05$ vs. values of respective control. $^{\#}p < 0.05$ vs. values for orlistat and cisplatin administered group. Pictures of mice (e) are from a representative experiment out of triplicates set with similar results. Arrows indicate change in body contour to reflect modulation of tumor growth.

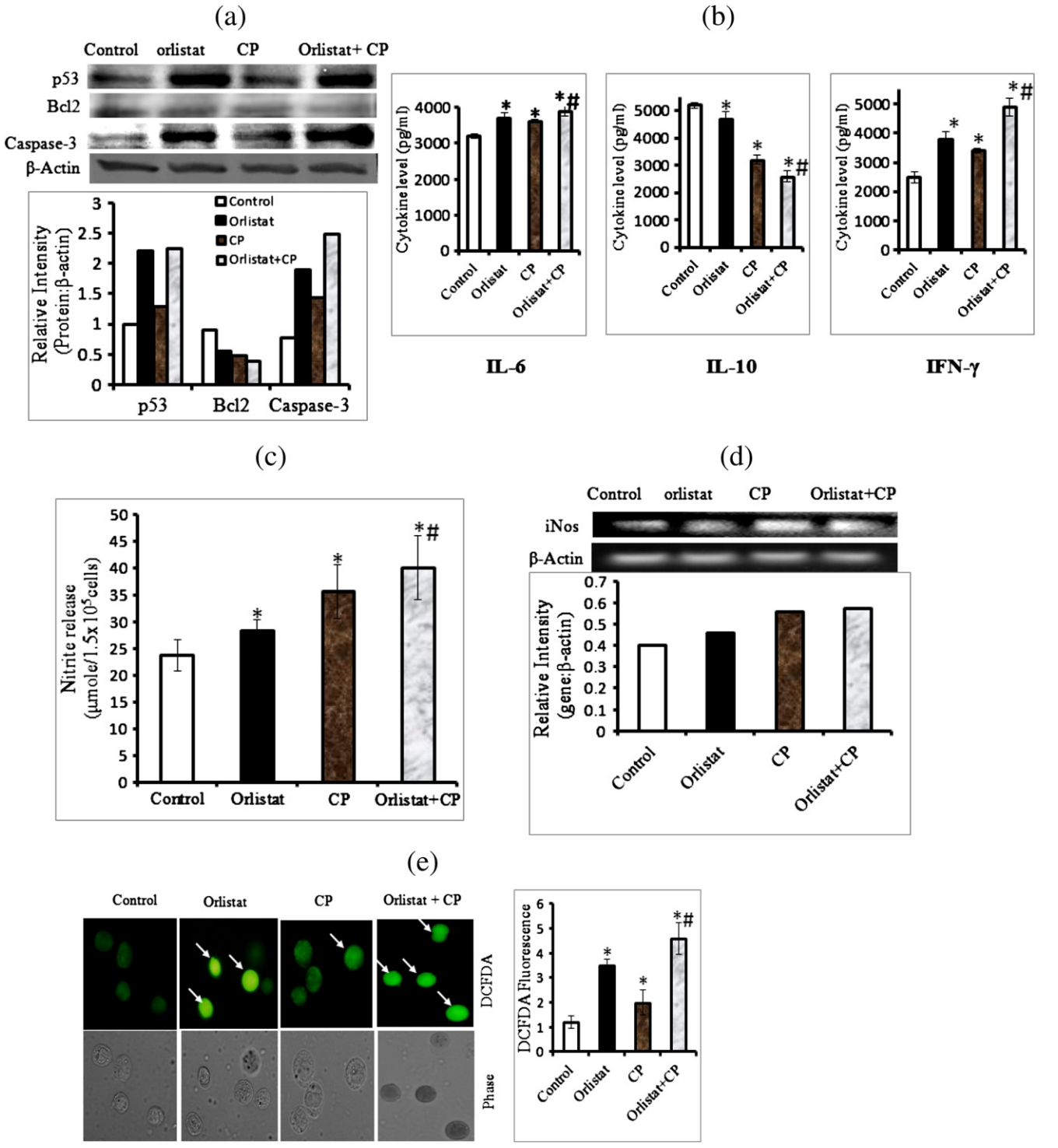

Fig. 3. Antitumor and chemosensitizing action of orlistat is accompanied by modulated expression of cell survival regulatory molecules. Tumor cells (1×10^6 cells) harvested from control and experimental groups were analyzed for expression of cell survival regulatory proteins (a). Ascitic fluid of these mice was examined for the level of indicated cytokines (b) and NO (c). iNOS (d) and ROS (e) as described in the Materials and methods. Values shown in (b, c, e) are mean ± SD of three independent experiments done in triplicate. Bands shown in (a) and (d), cells shown in plates (e) and their accompanying densitometric scans are from a representative experiment out of three independent experiments with similar results. $^{*}p < 0.05$ vs. values of respective control. $^{\#}p < 0.05$ vs. values for orlistat and cisplatin administered group.

3.3. Modulated FASN activity and expression following orlistat administration accompany augmented osmotic fragility of tumor cells

FASN activity (Fig. 4a) and expression of its gene (Fig. 4b) & protein (Fig. 4c) were analyzed in tumor cells of control and orlistat-administered mice in order to understand if FASN was inhibited following administration of orlistat to tumor-bearing host. The serum level of orlistat and its activity in tumor cells significantly declined in orlistat-administered group compared to control. Similarly, RT-PCR and western blot analyses indicated a decline in the expression

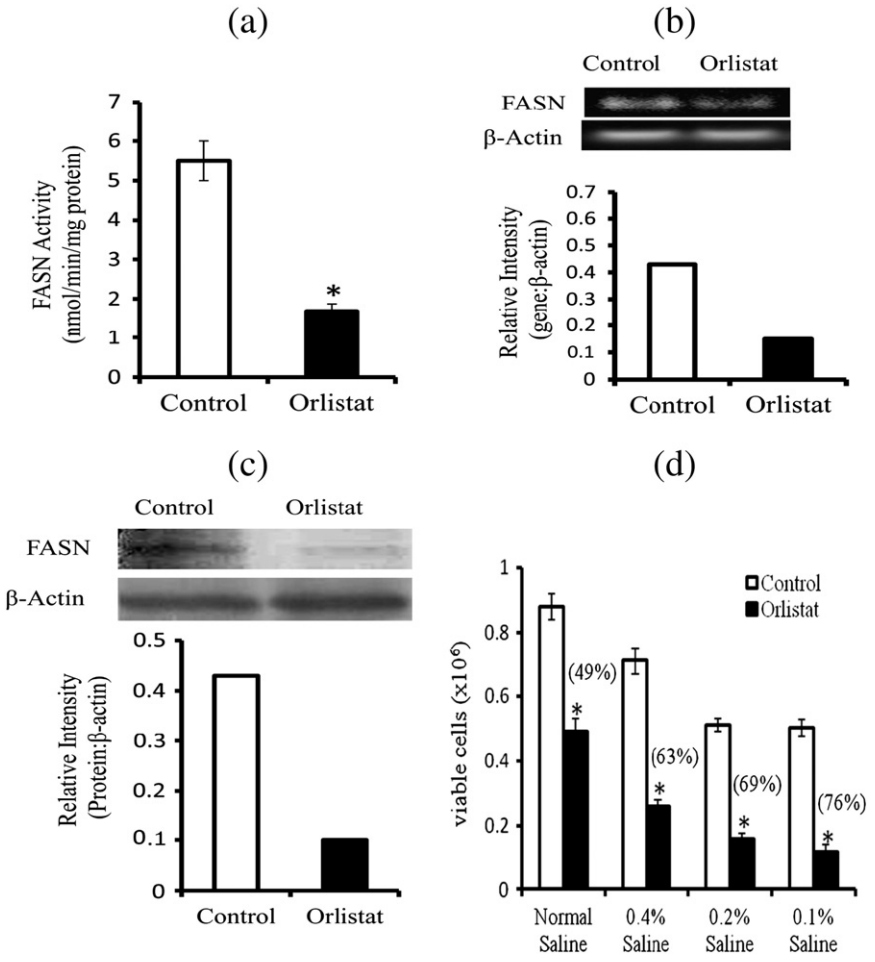

Fig. 4. Orlistat administration to tumor-bearing mice inhibits expression and activity of FASN in tumor cells associated with augmented osmotic fragility. Activity (a), expression of mRNA (b) and protein (c) of FASN in tumor cells obtained from control and orlistat-administered tumor-bearing hosts were estimated as described in the Materials and methods. Osmotic fragility of tumor cells harvested from control and orlistat-administered tumor-bearing hosts is shown (d). Values shown in parenthesis indicate % increase in osmotic fragility of orlistat group compared to their respective control (d). Bars shown in (b) & (c) are the densitometric scan of bands shown in the respective upper panel. Data shown in (b) & (c) is from a representative experiment out of three independent experiments with similar results. Values shown in (a) & (d) are mean ± SD of three independent experiments done in triplicate. $^{*}p < 0.05$ vs. values of respective control.

of the gene and protein of FASN in tumor cells harvested from orlistat-administered group. Declined FASN expression and activity are associated with declined membrane biosynthesis [15] and hence may affect the osmotic fragility of cells. Tumor cells harvested from orlistat-administered mice showed a significant increase in osmotic fragility compared to control (Fig. 4d).

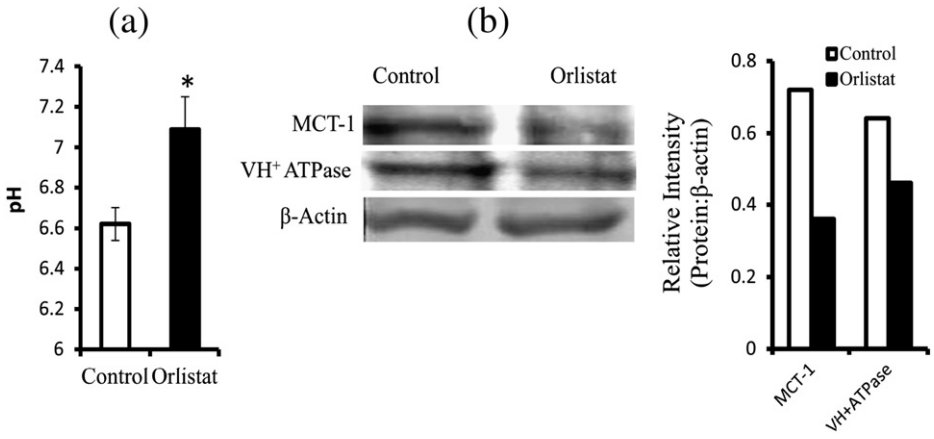

Fig. 5. Orlistat administration of tumor-bearing hosts alters pH homeostasis. pH of tumor ascitic fluid harvested from control and orlistat-administered tumor-bearing mice was examined (a). Tumor cells of control and orlistat group were immunoprobed by western blot for expression of pH regulators MCT1 and VH^{+} ATPase (b). Values shown in (a) are mean ± SD of three independent experiments done in triplicate. $^{*}p < 0.05$ vs. values of respective control of untreated cells. Bars shown in b are the densitometric scan of respective bands. Data shown in (b) is from a representative experiment out of three independent experiments with similar results.

3.4. Effect of orlistat-administration on pH of tumor ascitic fluid and expression of pH regulators

Since pH of tumor microenvironment plays a crucial role in regulating tumor cell survival and tumor progression, which in turn is mainly regulated by pH regulators expressed on plasma membrane of tumor cells, we checked if orlistat could alter pH homeostasis, which could be a critical contributor in its tumor growth retarding actions. Therefore, we also examined the pH of tumor ascitic fluid (Fig. 5a) and expression of two key pH regulators of tumor cells: monocarboxylate transporter-1 (MCT-1) and Vacuolar-type H^+-ATPase (VH^+ ATPase) (Fig. 5b). Orlistat administration to tumor-bearing mice resulted in a significant increase in the pH of tumor ascitic fluid compared to control. On the other hand the expression of MCT-1 and VH^+ ATPase proteins was found to decline in tumor cells harvested from orlistat-administered mice compared to respective controls.

3.5. Orlistat administration modulates the expression of multidrug resistance regulating proteins in tumor cells

Considering the fact that chemosensitivity of tumor cells is associated with altered expression of multidrug resistance regulating molecules [1,28], in the next part of the study we investigated the effect of orlistat administration to tumor-bearing mice on the expression of multidrug resistance protein (MDR) and multidrug resistance associated protein-1 (MRP-1). Tumor cells (1×10^6 cells/ml) harvested from control and orlistat-administered tumor-bearing mice were analyzed for the expression pattern MDR and MRP-1 by western blot. Orlistat administration manifested an inhibited expression of both of these proteins compared to their respective control (Fig. 6).

3.6. Orlistat primes tumor cells for augmented cisplatin-induced cytotoxicity

In order to understand if orlistat could directly prime tumor cells rendering them susceptible to the cytotoxic action of cisplatin, tumor cells (1×10^6 cells/ml) harvested from the untreated tumor-bearing host were incubated in vitro for 48 h in medium alone or containing orlistat (10 μM) followed by addition of cisplatin (1 μg/ml) for 24 h. As shown in Fig. 7 tumor cells incubated in the medium containing orlistat showed a significant inhibition of survival, which was further synergistically augmented following addition of cisplatin compared to that of cisplatin or orlistat alone.

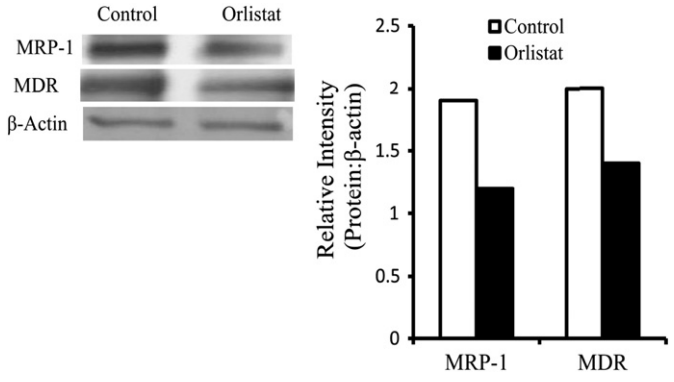

Fig. 6. Orlistat administration to tumor-bearing mice inhibits expression of MRP-1 and MDR proteins in tumor cells. Tumor cells (1×10^6 cells/ml) harvested from tumor-bearing mice administered with vehicle alone or containing orlistat were analyzed for protein expression of MRP-1 and MDR by western blot as described in the Materials and methods. Bars shown are densitometric scan of respective bands. Data shown is from a representative experiment out of three independent experiments with similar results.

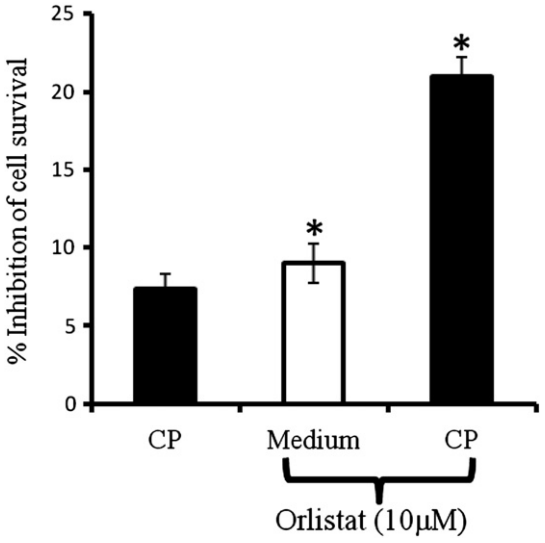

Fig. 7. Orlistat primes tumor cells for cisplatin-induced cytotoxicity in vitro. Tumor cells (1×10^6 cells/ml) were incubated for 48 h in medium alone or containing orlistat (10 μM). Tumor cells were then washed and incubated for 24 h in medium with or without cisplatin (1 μg/ml) followed by estimation of cell survival. Values shown are mean ± SD of three independent experiments done in triplicate. *$p < 0.05$ vs. values of respective control of untreated cells.

4. Discussion

The findings of the present investigation indicate that administration of orlistat to a murine host bearing a progressively growing T cell lymphoma renders dual therapeutic benefits by retarding tumor progression and augmenting the cytotoxic action of cisplatin. Increase in apoptotic tumor cell population following orlistat-administration indicates that induction of apoptosis could most likely mediate the antitumor action of orlistat in the tumor-bearing hosts. Moreover, the up-regulated expression of pro-apoptotic p53 and caspase-3 proteins accompanied by a decline of anti-apoptotic Bcl2 corroborates this notion. Apoptosis inducing action of orlistat has also been reported in a variety of other tumor cells [6–8,10,13,29,30]. Possibility of inhibited membrane biogenesis, following exposure of tumor cell to orlistat, rendering them vulnerable to induction of apoptosis could also constitute one of the main mechanism(s) of action of orlistat as in vivo administration of the drug augmented the osmotic fragility of tumor cells. Indeed, inhibition of FASN activity has been assigned as one of the attributory causes for induction of apoptosis owing to a declined membrane biogenesis [6,8–10,15]. Moreover, these osmotically fragile tumor cells are most likely to be susceptible to the cytotoxic action of cisplatin.

Considering the recent evidences that the components of tumor microenvironment play a pivotal role in regulation of tumor progression [4,5,18,31,32], we examined if orlistat administration to tumor-bearing host could also alter some of such key membrane dependent regulatory mediators of tumor microenvironment. Orlistat administration caused an up-regulated expression of iNOS associated with an increased generation of NO and ROS. Both NO and ROS have been implicated not only in induction of apoptosis in tumor cells but also in rendering tumor cells susceptible to the cytotoxic action of anticancer drugs [33–36]. Moreover, NO and ROS are also reported to modulate the expression of several cell survival regulatory molecules [37–39]. Interestingly, we also observed a decline in the level of IL-10 in tumor ascitic fluid accompanied by an elevation of IFN-γ. IL-10 is widely reported to be involved in tumor growth promotion [40–42], whereas, IFN-γ is implicated in retarding tumor growth, modulating expression of apoptosis regulating molecules [43–45]. Moreover, IFN-γ has also been reported to mediate orlistat-dependent inhibition of FASN [14,46,47]. We also observed alkalinization of tumor ascitic fluid following administration of orlistat

to tumor-bearing host, which could be an additional mechanism by which orlistat might manifest retardation of tumor growth and exert priming action for cytotoxicity of cisplatin. Indeed, tumor acidosis is widely reported to promote tumor growth [3,17,18]. Alkalinization of tumor microenvironment following orlistat administration to tumor-bearing host could be a consequence of an inhibited expression of membrane associated pH regulators: MCT-1 and VH^+ ATPase. Indeed both MCT-1 and VH^+ ATPase play a central role in regulating tumor acidosis [18,22,48]. The reason for the declined expression of these pH regulators could be attributed to a declined membrane biosynthesis. It is also likely that declined tumor load following orlistat-administration could also play a contributory role in reversal of tumor acidosis.

Another important observation of this study showed that the exposure of tumor cells to orlistat in vivo inhibits expression of MRP-1 and MDR proteins, which are implicated in manifestation of MDR phenotype in tumor cells [28,49,50]. Thus this could be implicated as one of the mechanisms making the tumor cells susceptible to cisplatin cytotoxicity. Nevertheless, the expression of MDR is reported to be under the regulatory control of altered pH and cytokines [51–54]. Moreover, NO and ROS have also been implicated in regulation of MDR phenotype of tumor cells [55,56]. The declined expression of MDR could thus be a result of an interplay of multiple triggering factors generated in tumor-microenvironment following administration to orlistat in vivo.

Taken together the observations of this study report for the first time that exposure of tumor cells in vivo to orlistat manifests antitumor activity and also exert chemosensitizing action on tumor cells, for cytotoxicity of cisplatin. This implicates an array of molecular events culminating in a reconstituted tumor-microenvironment and decline of MDR phenotype. A summary presenting the mechanism(s) of orlistat-dependent tumor growth retardation and augmentation of the cytotoxic action of cisplatin is presented in Fig. 8 depicting the involvement of modulated expression of cell survival regulatory molecules, reconstituted tumor-microenvironment, altered FASN activity & expression rendering tumor cells osmotically fragile and altered MDR phenotype. These observations will be of immense help in optimizing the use of orlistat as an adjunct regimen for optimizing the therapeutic efficacy of mainstream anticancer drugs.

Acknowledgement

The financial support to the School of Biotechnology from DBT, UGC University with potential for excellence program and the DST-PURSE program, Govt of India, New Delhi is acknowledged. The authors express gratitude to DBT and CSIR New Delhi, for fellowship support to Shiva Kant (DBT-JRF/2010-11/79) and Ajay Kumar (09/013(0329)/2010-EMR-I) respectively. The author expresses gratitude to Dr. Syamal Roy (IICB Kolkata) for providing antibodies against MDR and MRP-1 proteins.

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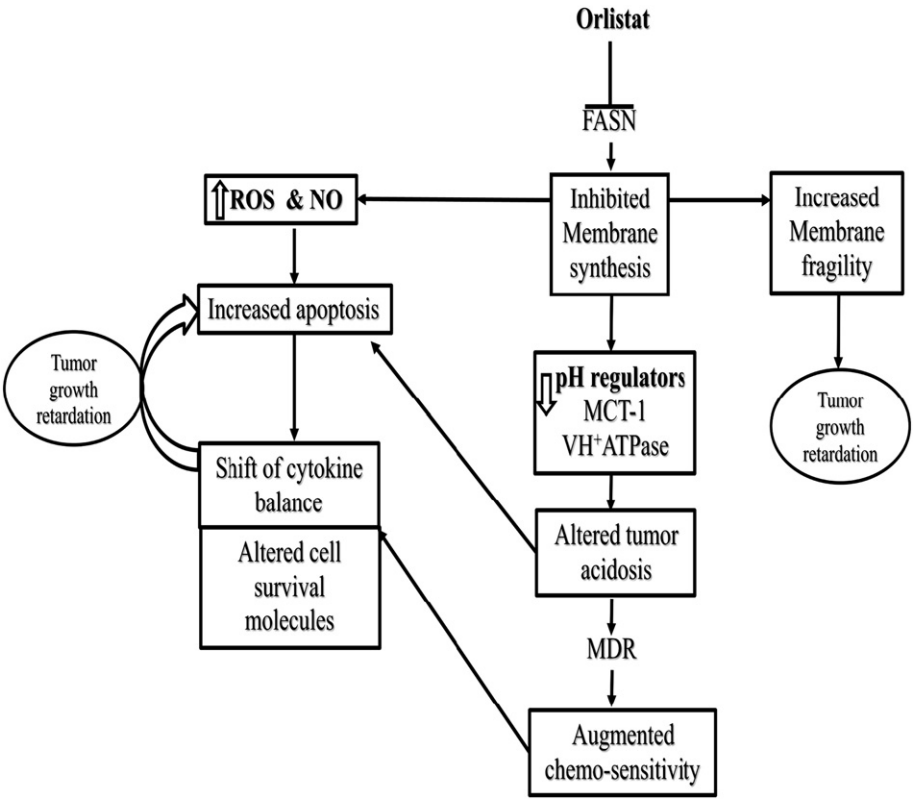

Fig. 8. Possible mechanism(s) and mediators of orlistat dependent tumor-growth retardation and chemosensitization. The figure depicts that inhibitors of FASN by orlistat administration to tumor-bearing mice ushers tumor growth retardation associated with declined tumor cell survival, augmented apoptosis, increased membrane fragility, altered pH homeostasis and reconstituted tumor microenvironment rendering tumor cells chemosensitized to cytotoxicity of cisplatin.

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