RESEARCH ARTICLE

Bag-1L is a Stress-withstand Molecule Prevents the Downregulation of Mcl-1 and c-Raf Under Control of Heat Shock Proteins in Cisplatin Treated HeLa Cervix Cancer Cells

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Abstract

Background: Cisplatin, a DNA damaging agent, induces apoptosis through increasing DNA fragmentation. However, identification of intrinsic resistance molecules against Cisplatin is vital to estimate the success of therapy. Bag-1 (Bcl-2-associated anthanogene) is one anti-apoptotic protein involved in drug resistance impacting on therapeutic efficiency. Elevated levels of this protein are related with increase cell proliferation rates, motility and also cancer development. For this reason, we aimed to understand the role of Bag-1 expression in Cisplatin-induced apoptosis in HeLa cervix cancer cells. Cisplatin decreased cell viability in time- and dose-dependent manner in wt and Bag-1L+HeLa cells. Although, 10μM Cisplatin treatment induced cell death within 24h by activating caspases in wt cells, Bag-1L stable transfection protected cells against Cisplatin treatment. To assess the potential protective role of Bag-1, we first checked the expression profile of interacting anti-apoptotic partners of Bag-1. We found that forced Bag-1L expression prevented Cisplatin-induced apoptosis through acting on Mcl-1 expression, which was reduced after Cisplatin treatment in wt HeLa cells. This mechanism was also supported by the regulation of heat shock protein (Hsp) family members, Hsp90 and Hsp40, which were involved in the regulation Bag-1 interactome including several anti-apoptotic Bcl-2 family members and c-Raf.

Keywords: Bag-1 - Hsp family - cervical cancer - apoptosis - drug resistance

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Introduction

Cervical cancer affects the women worldwide and it is the second most common cancer remaining a major health problem because of high oncogenic human papilloma virus (HPV) infection rates (Packham et al., 1997). Most women in developed countries are diagnosed at early stages of disease, which is cured with only surgery. However, treatment success is limited in recurrent cases with risk ratios as 10-20% for International Federation of Obstetrics and Gynecology (FIGO) stages Ib-IIa and in locally advanced cases it is 50-70% (stages IIb-IVa) (Rushdan et al., 2004). Classical chemotherapeutics are performed alone or in combination with other drugs or therapy strategies to treat recurrent cervival cancer cells (Zighelboim et al., 2013; Zhang et al., 2014). Platinum-based chemotherapy has recently proved to be an effective therapy for cervical cancer. Cisplatin (DDP), a platinum based chemotherapeutic agent, activates death mechanisms in the cell (Newman and Cragg 2004). Cisplatin is cis-diamminedichloroplatinium (II) was discovered at 1960s and has been used as platinum-based chemotherapeutic agent in the treatment of different cancer types such as; breast, ovary, testes, head and neck cancers (Roy and Mukherjee 2014).

Bag-1 (Bcl-2 associated anthanogene) gene, is located on human chromosome 9, band 17, contains 7 exons, encodes 3885 base pair long mRNA (Takayama et al., 1995). The multifunctional Bag-1 protein interacts with a wide range of cellular targets and regulates cell survival, signaling, metastasis, proliferation, and transcription mechanisms in the cells. Cells express several Bag-1 isoforms through alternate translation initiation of a single mRNA. The most abundant isoform, p36 Bag-1S, is translated from an AUG codon and is predominantly a cytoplasmic protein. The largest isoform, p50 Bag-1L, is translated from an upstream CUG codon and, consistent with the presence of a nuclear localization signal (NLS) within its NH2-terminal extension, resides within the cell nucleus. The other isoform, p46 Bag-1M, is generally expressed at low levels in human cells and is not detected in other species (Cutress et al., 2002). Various domains have been recognized within Bag-1 isoforms, including ubiquitin-like domain (ULD) and a COOH-terminal

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evolutionarily conserved BAG domain. Bag-1 protects cells from a wide range of apoptotic stimuli, including Fas, cytotoxic drugs, staurosporine, heat shock, and growth factor withdrawal.

Bag-1 overexpression has an effective survival role in the development and response to several drugs in various cancer types such as breast, lung, prostate, thyroid and gastrointestinal cancer. Elevated level of Bag-1 protein was observed in cervival cancer development (Hassumi-Fukasawa et al., 2012). According to the cervival cancer outcomes, higher expression of Bag-1 and Bcl-2 were determined in cervical carcinoma tissues rather than normal tissues. Bag-1 has a role to inhibit apoptosis via MAPK cascade activating Ras, Raf-1, MEK, ERK signaling molecules. In addition, Bag-1 has been observed in p53 mutant cancer cells and causes suppression of p53-mediated apoptosis (Townsend et al., 2003; Zheng et al., 2010).

The anti-apoptotic Bag-1 protein, which does not belong to Bcl-2 family, binds and interacts with the Bcl-2 protein and enhances the anti-apoptotic ability of Bcl-2 protein. The Bag-1 COOH terminus is also required for interaction and activation of Raf-1, a serine-threonine kinase involved in cell survival and proliferation that provides an alternate potential mechanism by which Bag-1 might promote cell survival. Hsp70 and Raf-1 compete for binding to Bag-1, and high levels of Hsp70 prevent activation of Raf-1 by Bag-1 in hamster fibroblasts, suggesting that growth and/or survival-promoting effects of Bag-1 may be mediated by activation of Raf-1-dependent mitogen-activated protein kinase pathways and negatively regulated by chaperone binding (Zheng et al., 2010). Apart from interactions with Bcl-2, Bag-1 serves as a co-chaperone to Hsp/Hsc70s by interacting with the ATPase domain of these proteins (specifically IB and IIB subdomains) to exchange ADP for ATP under normal and stress conditions, positively affecting the activity of Hsp70 system. Recruitment of Bag-1 by Hsp/Hsc70s can, in one line, link this complex to the E3 ligases of the proteasome machinery such as Siah, CHIP; thus causing the activation of ubiquitin-dependent proteolytic rescue system of the cell and, consequently, regulate the balance between apoptosis and survival (House et al., 2006). Under stress conditions, the acute need for chaperones elevates Hsp70 levels, and this can cause most of Bag-1 molecules in the cell to be more involved in either the correct folding of unstable substrates or their targeting to the proteasome machinery for degradation. Under normal conditions though, some number of Bag-1 proteins can be freed to get involved in signaling processes. In addition, direct interaction with Hsp70/Hsc70 system can link Bag-1 isoforms to c-Jun, which is a component of a transcription factor complex, Activator Protein-1 (AP-1). c-Jun acts as an oncoprotein in collaboration with an another oncoprotein, c-Fos, by elevating transcription and protein synthesis thus leading to cancer development (Fralix et al., 2003).

Bag-1 is involved in both cell survival and apoptosis major pathways, but the molecular details of these mechanisms and crosstalk between the pathways are not clarified yet. In this study, we aim to understand the involvement of Bag-1 in cell decision between apoptosis and survival. For this reason, we investigated the effect of anti-apoptotic Bag-1 protein expression level in the cisplatin-induced apoptosis and drug resistance mechanism. We target the induction of apoptosis via cisplatin treatment in contrast; we overexpressed Bag-1 protein to study its role in the drug resistant apoptotic pathway in HeLa cells.

Materials and Methods

Cell culture

Cervix cancer cell lines, HeLa (CCL-2; ATCC, Manassas, VA, USA) and Bag-1L stable transfected HeLa cells (produced with of pEZ-M02-Bag-1L plasmid DNA vector and stable colonies were selected by Neomycin (Gibco-Life Technologies, CA, USA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco-Life Technologies, CA, USA) with 10% (v/v) heat inactivated fetal calf serum (Pan Biotech, Aiedenbach, Germany) and penicillin-streptomycin (10,000 units penicillin/ml, 10 mg streptomycin/ml) (Pan Biotech, Aiedenbach, Germany) at 37˚C in a humidified 5% CO2 incubator (Hera Cell 150i, Thermo, San Jose, CA, USA).

MTT cell viability assay

HeLa wt and Bag-1L stable transfected cells were seeded at $1X10^4$ density in 96-well plates (TPP Zellkultur Testplatte, Sweden) and treated with various concentrations of Cisplatin (0-50μM) for 24h and 48h and both cell lines are treated 10μM Cisplatin (Koçak Pharma, 10mg/20ml, stock concentration 1mM) in time-dependent manner. Cells were exposed to 10μl of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide dye (Sigma, Life Sciences, USA, final concentration: 5 mg/ml) and were incubated at 37˚C (Heraeus, Hera Cell 150; Thermo) for 4h for the conversion of MTT to MTT-formazan crystals by mitochondrial enzymes. After that, 100μl DMSO (Sigma, Life Sciences) is added to cells for solubilization of formazan crystals. Absorbance was determined at 570 nm spectrophotometrically (Model 680 Microplate Reader Bio-Rad, Hercules, CA, USA).

Trypan blue dye exclusion assay-survival assay

HeLa wt and Bag-1L stable transfected cells were seeded at 1×10^5 density in 6-well plates (TPP Zellkultur Testplatte) and treated with 10μM Cisplatin at every 24h within 96h. First, cells were trypsinized (Trypsin EDTA (0.25%), Gibco-Life Technologies) and centrifugated then, cells were exposed to 0.4% (w/v) Trypan Blue (Gibco-Life Technologies) (50μl) and DMEM (50μl) at 1:1 ratio. After that, 10μl of cells were counted by dual-chamber 0.1mm deep Neubauer improved haemocytometer (Marienfield Superior, Germany). Viable and non-viable cells were recorded and based on viable cells a graph is formed.

Detection of apoptotic cells by flourescent microscopy

HeLa wt and Bag-1L stable transfected HeLa cervix cancer cells were seeded at a density 1×10^5 in 6-well plates and treated with 10μM Cisplatin for 24 and 48h. Then, cells were washed with 1×PBS (Pan Biotech, Aiedenbach, Germany) and exposed to

propidium iodide (PI) (stock concentration: 50mg/ml, final concentration: 1μg/ml; Applichem, Darmstadt, Germany), 3,3'-dihexyloxacarbocyanine iodide (DiOC6) (stock concentration: 4 mM, final concentration: 2 µg/ml, Fluka by Sigma-Aldrich, MO, USA) and 4',6-diamidino-2-phenylindole; DAPI (Molecular Probes, Eugene, OR, USA) for 30, 15 and 5 minutes, respectively. After that, cells were washed with 1x PBS and images were taken by fluorescent microscopy (I×70; Olympus, Japan)

Immunoblotting analysis

HeLa wt and Bag-1L stable transfected cells were treated with 10μM Cisplatin time-dependent manner (0-72h). Then, cells were washed with ice-cold 1x PBS and lysed in ProteoJET Mammalian cell lysis reagent (Fermantas, Hanover, MD, USA) with protease inhibitor cocktail. After lysis, cells were centrifugated for 15 min. at 13200 rpm and total protein concentrations were determined with Bradford protein assay (Bio-Rad, USA). Then, 30 μg total protein was seperated on 12% sodium dodecyl sulphate (SDS)-polyacrylamide gels and transferred difluoride (PVDF) membranes (Roche, USA). Following the washing of membranes in tris-buffered saline with Tween-20 (TBS-T) (10mM Tris-HCl (pH 8), 0.05% Tween-20) (Tween20: Sigma-Aldrich) and they were blocked by 5% skim milk containing TBS-T milk overnight at 4˚C. Then, PVDF membranes were incubated in primary antibody buffer containig 5% (v/v) skim milk solution (cleaved Caspase-7 (1:1000), cleaved Caspase-9 (1:1000), Bcl-xL (1:1000), Mcl-1 (1:1000), Bag-1 (1:1000), Bcl-2 (1:1000), c-Raf (1:750), Ras (1:1000), Hsp90 (1:1000), Hsp70 (1:1000), Hsp60 (1:1000), Hsp40 (1:1000), HSF-1 (1:1000), β-actin (1:1000); (Cell Signaling Technology, Danvers, MA, USA) overnight at 4˚C. Then membranes were rinsed with TBS-Tween 20 and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit IgG or anti-mouse IgG (Cell Signaling Technology) secondary antibodies for overnight at 4˚C. Membranes were developed with an enchanced chemiluminescence reagents (Lumi-LightPLUS Western Blot substrate, Roche, USA) and exposed to Hyperfilm-ECL (Hyperfilm-ECL, Amersham Pharmacia Biotech, Freiburg, Germany). All proteins were quantified relative to the loading control β-actin.

Statistical analysis

All the experiments were statistically analyzed by a two-way ANOVA using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Statistically significant results by ANOVA were further analyzed by Bonferroni post-hoc analysis (where indicated). A p-value<0.05 was considered statistically significant. Error bars in the graphs were generated using±standard deviation (SD) values. Western Blot results were repeated at least twice and Image J program was applied to get band intensities.

Results

Bag-1L prevented cisplatin-induced apoptosis in HeLa cells

HeLa wt and Bag-1L stable transfected cells were treated with various concentrations of Cisplatin (0-50 µM) for 24 and 48h and then MTT cell viability assay was utilized to determinethe cytotoxicity. Cisplatin (10µM) treatment for 24h caused a sharp decrease in relative cell viability ratio by 40% in wt HeLa cells (Figure 1A-B). This sharp decrease was found significant. Therefore, the concentration was chosen as an effective cytotoxic dose for further experiments. Contrary, Cisplatin treatment for 24h only decreased cell viability by 25% in Bag-1L stable transfected HeLa cervical cancer cells. We determined that Cisplatin treatment time-dependently decreased cell viability wt and Bag-1+HeLa cells. Therefore we concluded that, Cisplatin-induced cytotoxicity could be attenuated by Bag-1L stable transfection, which prevented cisplatin-induced cell death.

To estimate the promoting role of Bag-1 on cell survival, cells were stained with Trypan Blue dye and then non-stained cell populations were counted on a haemocytometer after Cisplatin (10µM) treatment at different time points (24, 48, 72 and 96h). As shown in Figure 2A while untreated control cells were growing exponentially every 24h, Cisplatin decreased the number of viable cells time-dependently in wt HeLa cells. Bag-1L stable transfected cells were resistant against Cisplatin

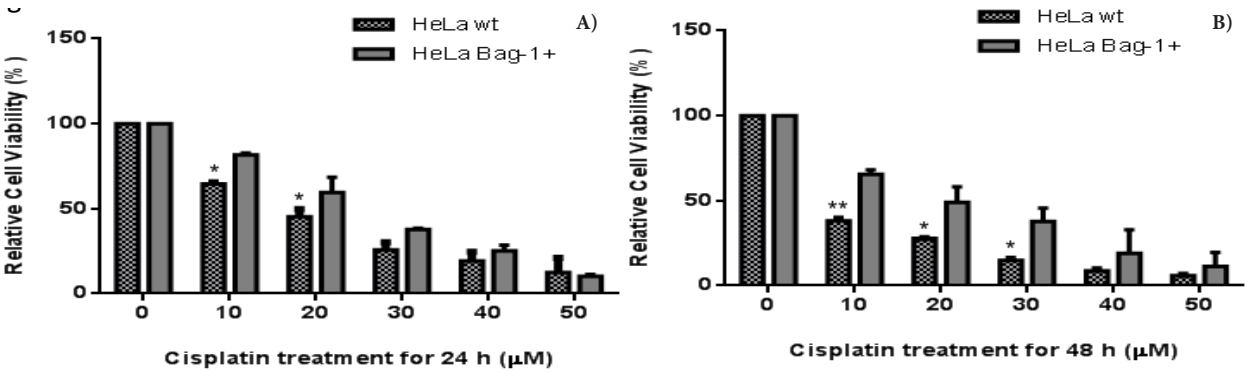


Figure 1. Cisplatin Decreased the Cell Viability in a Dose and Time Dependent Manner in Wild Type (wt) and Bag-1L Stable Transfected HeLa Cells. Cells were seeded at 1×10^4 density and treated with various concentrations of Cisplatin (0-50µM) for 24 and 48h (A-B). The bar histograms represent the mean±SD of four independent trials with at least four replicates. A) p<0.0001, B) p<0.0001 each result was compared with the untreated control cells by using Bonferroni's ANOVA 95% multiple comparison test)

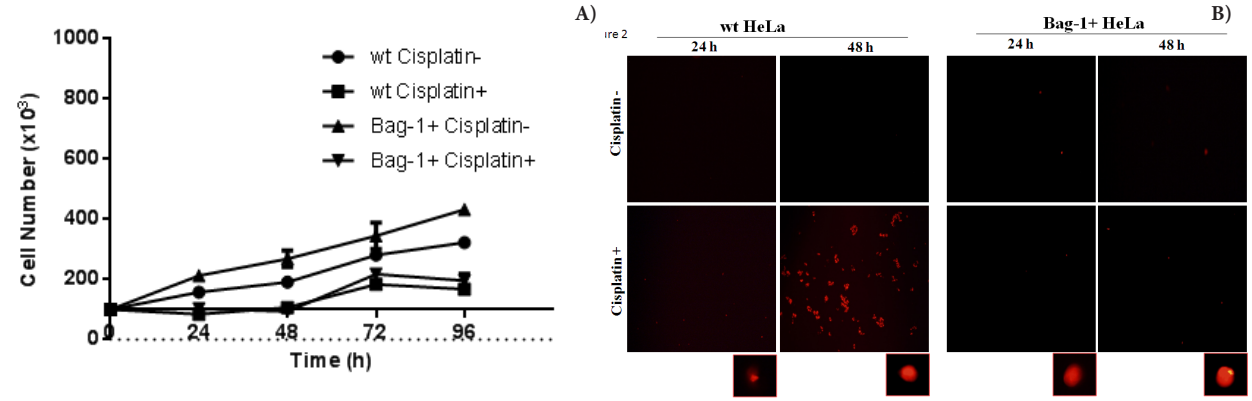


Figure 2. A) Bag-1L Stable Overexpression Enhanced the Protection Effect Against Cisplatin in HeLa Cells Proliferation Rates. wt and Bag-1L+ cells were seeded at 1×10^6 density in 6-well plates and treated with 10μM Cisplatin for 24,48,72 and 96h, According to the time points, cells counted each day for 4 days using trypan blue dye exclusion assay. Lines represent unstained viable cell numbers and the means±SD of three independent trials with at least four replicates; **B) Bag-1L Stable Transfection Decreased Dead Cell Number in Cisplatin-induced Apoptosis in wt and Bag-1L+HeLa Cells.** Cisplatin-treated apoptotic cells were determined as red dots by PI staining. Images were taken at 200× expansion of cells

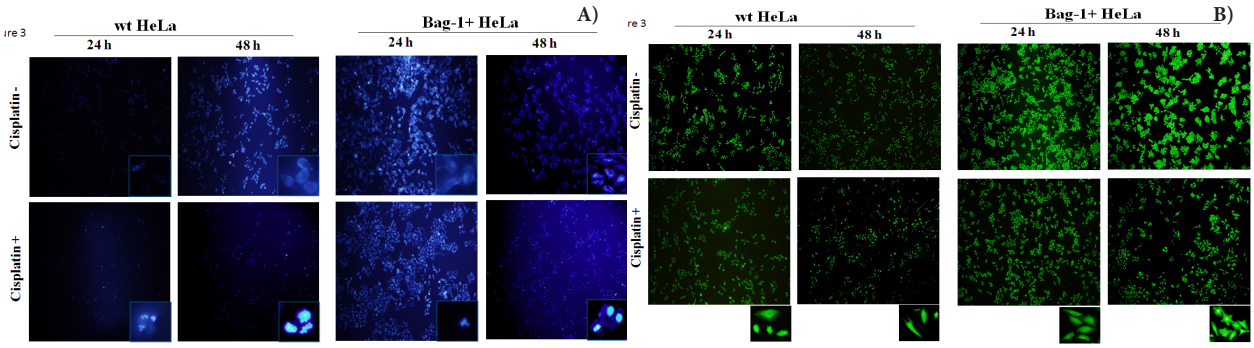


Figure 3. A) Determination of MOMP was Performed Following Cisplatin Treatment in wt and Bag-1L+HeLa Cells. The Loss of MOMP was Determined by DiOC6 Staining. Images were taken at 200× expansion of cells; **B) Determination the Effect of Cisplatin in wt and Bag-1L+HeLa Cells was Observed via DAPI Staining.** Bright blue dots were determined the DAPI stained apoptotic cells by fluorescence microscopy. Images were taken at 200× expansion of cells

treatment compared to wt HeLa cells.

Since cell membrane of healthy cells is not conductive to PI staining, we checked the protective role of Bag-1 in Cisplatin-induced cell death mechanism. Cells were stained with PI for 15min after drug treatment for 24 and 48h (Figure 2B). In accordance with previous MTT cell viability data, PI stained positive cell number were increased after drug treatment time-dependently in HeLa wt cells. However, Bag-1 stable transfected cells were more resistant compared to wt HeLa cells against Cisplatin treatment.

Bag-1 augmented resistant phenotype of HeLa cells against Cisplatin by altering mitochondria-mediated apoptosis

DiOC6 fluorescent dye joins to the mitochondria membrane; but it does not bind to mitochondria membranes, which have lost mitochondrial integrity as a result of the activation of apoptosis signaling cascade. As shown in Figure 3A Cisplatin treatment caused a significant decrease in DiOC6 staining ratio in wt HeLa cells but similar to previous findings of this study, Bag-1L stable transfection protected cells against Cisplatin-induced loss of mitochondrial membrane potential. To estimate the DNA damaging effect of Cisplatin, cells were stained with DAPI, which stains DNA and causes a blue fluorescent beam helping the observation of DNA fragmentation as result of apoptosis. As shown in Figure 3B the bright blue nuclear stained cell number was higher after Cisplatin treatment compared to untreated cells. However, Bag-1L stable transfection was only effective to render apoptotic efficiency of Cisplatin for 24h.

Bag-1L overexpression protected cells against Cisplatin-induced apoptosis by increasing Mcl-1 expression in HeLa cells

In order to establish the protective role of Bag-1L in HeLa cells against Cisplatin-induced apoptosis, we first checked caspase activation by detecting cleavage profile of Caspase-9 and Caspase-7. After exposure of wt and Bag-1L+HeLa cells to Cisplatin time-dependently, we isolated total protein content and proceeded immunoblotting assay. As shown in Figure 4 although Cisplatin-induced cleavage of Caspase-9 and Caspase-7 in both cell lines, long-term treatment of Cisplatin for 72h caused a sharp increase in cleaved product of Caspase-9 in HeLa Bag-1L+cells.

Cisplatin treatment for 48h effectively downregulated Bag-1L and Bag-1M expression levels and it was only effective on Bag-1S expression profile after 72h treatment. The plasmid-mediated Bag-1L expression efficiency was higher in stable transfected HeLa cells. Interestingly, not only Bag-1L constructs the expression

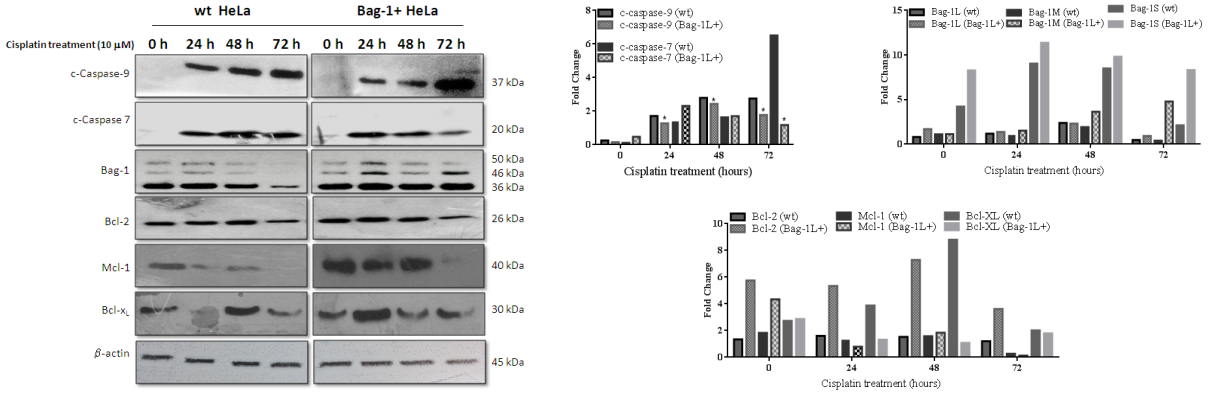


Figure 4. Cisplatin induced Apoptosis Via Modulating Caspases and Bcl-2 Family Members was Observed by Immunoblotting. Cells were seeded at 1×10^6 density in 60mm plates and treated with Cisplatin for 24, 48 and 72h. A total 30μg of protein lysate was loaded in 12% SDS page gel. β-actin was performed as a loading control. Image J data analysis for immunoblotting assay was given as column graphs for each protein in wt and Bag-1L+HeLa cells

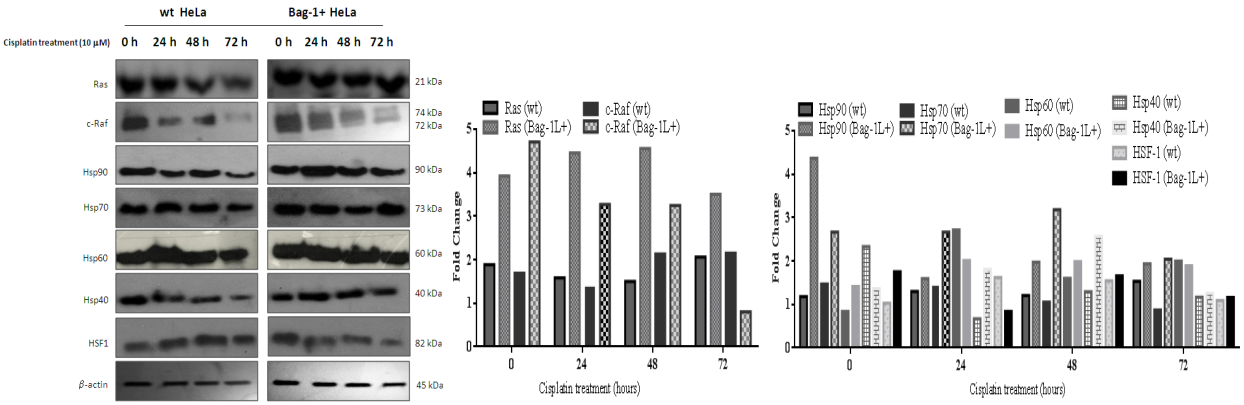


Figure 5. The Effect of Bag-1L Stable Transfection on Oncogenes and Heat Shock Proteins in wt and Bag-1L+HeLa Cells was Observed by Immunoblotting. Cells were seeded at 1×10^6 density in 60mm plates and treated with Cisplatin for 24, 48 and 72h. A total 30μg of protein lysate was loaded in 12% SDS page gel. β-actin was performed as a loading control. Image J data analysis for immunoblotting assay was given as column graphs for each protein in wt and Bag-1L+HeLa cells

levels of Bag-1M and Bag-1S isoforms were higher in the presence of Cisplatin. According to time-dependent immunoblotting assay determinations, Cisplatin treatment for 24h downregulated only Bag-1L and Bag-1M isoforms but not Bag-1S in wt and Bag-1L+HeLa cells. This effect was reversed when the cells were long-term treated with Cisplatin for 48h. Exposure of cells with Cisplatin for 72h downregulated Bag-1L and Bag-1M expression, whereas it did not effect on Bag-1S expression levels. Starting from this point, we concluded that stable transfection of Bag-1L guards cells from drug-induced apoptosis in HeLa cells every 24h. The expression level of Bcl-2, binding partner of Bag-1, was not altered after Cisplatin treatment at different time points within 72h in wt and Bag-1L+HeLa cells. However, Bag-1L stable transfection prevented drug-induced downregulation of Mcl-1 and Bcl-xL within 48h in HeLa cells. Long-term exposure of Bag-1L+HeLa cells with Cisplatin for 72h was effective to downregulate Mcl-1 and Bcl-xL expression levels.

Bag-1 promotes heat shock proteins and Raf/Ras signaling to protect cells against Cisplatin-induced apoptosis

The proposed interaction partners of Bag-1L were Ras and c-Raf, proto-oncogenes involved in the regulation of cell death, proliferation and survival processes in the cells were also investigated after Cisplatin treatment in wt and Bag-1L+HeLa cells. According to findings, Cisplatin downregulated c-Raf expression within 24h but later showed suppressing role on Ras in wt HeLa cells. Bag-1 stable transfection prevented Cisplatin-induced downregulation of Ras and c-Raf expression.

To understand the potential effect of Bag-1 interaction partners in Cisplatin-induced apoptosis mechanism, we also checked the expression profile of Hsp family after Cisplatin treatment in wt and Bag-1L+HeLa cells (Figure 5). When the cells were exposed to Cisplatin, the expression level of Hsp70 was downregulated time-dependently in wt HeLa cells compared to untreated control samples. Concomitantly, the expression level of Hsp40, which is a co-chaperone of Hsp70, was downregulated. Contrary to these observations, Bag-1L stable transfection caused a significant upregulation for Hsp70 and Hsp40 expression levels although cells were exposed to DNA damaging drug. Additionally, Bag-1L stable transfection postponed Cisplatin-induced downregulation of Hsp60, a pro-apoptotic chaperone. Hsp90 expression level was also promoted within 24h through Bag-1L stable transfection in the presence of Cisplatin, whereas exposure of wt HeLa cells to Cisplatin downregulated Hsp90 expression levels. Bag-1L forced expression reversed upregulatory effect of

Cisplatin on HSF1 expression. Thus, we concluded that Bag-1L expression regulates the Hsp family under stress conditions to prolong cell survival through acting on Mcl-1.

Discussion

Mostly, current anticancer strategies provoke cell death by altering different molecular players sequentially. However, the effect of therapeutics depend on the intrinsic factors, which eliminate the cell death inducing effect due to modulation of specific molecular targets under the control of environmental or hereditary factors. Therefore, intrinsic or acquired resistance factors may render the therapeutic efficiency in cancerous cells (Jamshed et al., 2014). Although, Cisplatin, as a strong apoptotic inducer through generating DNA breaks and led to apoptosis in cancer cells, some key proteins may prevent Cisplatin-induced apoptosis (Song et al., 2014). Recent studies suggested that silencing of Bag-1 enhanced the sensitivity against Cisplatin treatment through modulating Bcl-2 family members in A549 and L9981 lung cancer cell lines (Liu et al., 2010). It is suggested that Bag-1 is a bridge molecule between molecular chaperons, Hsps and growth signaling cascade to maintain cell proliferation (Townsend et al., 2003). In addition, overexpression of Bag-1 promotes cell survival and differentiation via interacting Bcl-2, an antiapoptotic protein. It is hypothesized that Bag-1 binds to Hsp/Hsc70-substrate complexes to regulate the potential substrates of this complex such as Bcl-2, Raf, NHRs (Wang et al., 1996; Wang and Reed 1998; Dobbyn et al., 2008). In order to assess the potential role of Bag-1 in drug-induced apoptosis mechanism, we questioned the role of several key players in cellular cell death and survival decision. According to MTT cell viability assay Cisplatin induced cell viability loss in dose and time-dependent manner in HeLa cervix carcinoma cells (Figure 1A-B). However, stable transfection of Bag-1L plasmid prevented cytotoxic effect of Cisplatin. In addition, the proliferation ratio of Bag-1L+ HeLa cells was more rapid than wt HeLa cells. Thus, Bag-1L plasmid transfected cells were more resistant to Cisplatin treatment than wt HeLa cells. These all findings proved that Bag-1L prevented Cisplatin-induced cell death and enhanced proliferation (Figure 2A-B). Similar results were shown in non-tumorigenic human colorectal carcinoma derived S/RG/C2 cells after γ-radiation or vitamin D analogue EB1089 treatment (Barnes et al., 2005). Nuclear localization of Bag-1L isoform enhanced cell survival and prevented apoptosis triggered by both therapeutic strategies. Nuclear localization of Bag-1, which provoke tumorigenesis was also associated with poor prognosis of esophagus carcinoma patients (Noguchi et al., 2003). The crucial role of Bag-1 for the survival and differentiation of hematopoietic and neuronal cells has been confirmed by Bag-1 knock-out experiments in mice. Bag-1-/-mice demonstrated higher rate of apoptosis in the fetal liver and developing nervous system. On the other hand, no significant alteration was determined at extracellular survival signaling cascade; Akt, Raf or their phosphorylated targets. Thus, Bag-1 was suggested as a physiological mediator of extracellular survival signals linked to the cellular mechanisms that prevent apoptosis in hematopoietic and neuronal progenitor cells (Gotz et al., 2005). Therefore, anti-apoptotic role of Bag-1 depends on its regulatory interacting partners, which structurally complementary (Zhou et al., 2014). Supporting role of Bag-1 in a close relation with Bag-3 was also determined in myeloid cell proliferation. Downregulation of both genes restricts cell survival by activating proteosome-derived degradation of main pro-survival Bcl-2 family members, directly or indirectly. In association with this observation, we found that when wt HeLa cells were exposed to Cisplatin (10μM), not Bcl-2 but Mcl-1 and Bcl-xL were downregulated within 24h (Figure 3). However, we did not determine same effect in Bag-1+ HeLa cells. The regulator of Mcl-1 expression is USP9X (Schwickart et al., 2010), was suggested an interacting partner of Bag-1, thus prevented Mcl-1 downregulation (Aveic et al., 2011) in HL60, NOMO1, NB4, THP1, MV4;11, and ML2 myeloid cells. In this point, we concluded that Bag-1 stable transfection prevented Mcl-1 downregulation due to proteosomal regulatory role of Bag-1 after Cisplatin treatment. In accordance, Bag-1 was reported to play an important role in protein folding and degradation via its interaction with Hsp70 and with the proteosome by the ubiquitin-like region, respectively (Takayama et al., 1997; Takayama et al., 1998). Recent studies indicated that expression levels of Bcl-2 was determinative the regulation of Bag-1 isoforms. Bag-1L stable transfection did not alter Bcl-2 expression whether cells were exposed to Cisplatin at different time points in HeLa cells. Although, forced Bag-1 expression supported cell proliferation and prevented Cisplatin-induced cytotoxicity, cleavage profile of Caspase-9 and Caspase-7 were not different due to Cisplatin treatment in both HeLa cell lines. We hypothesized that Bag-1 played a supportive role in cell proliferation and reduced apoptotic potential of Cisplatin through increasing expression levels of survival factors. To understand the modulation of Bag-1L interactome after Cisplatin treatment, Ras and Raf expression levels were determined in wt and Bag-1L+ HeLa cells (Figure 4). Bag-1 promoted survival after stress requires the precise orchestration of cell signalling events were required to ensure alerted biosynthetic processes and initiation of cell survival pathways (Song et al., 2001). It was suggested that Bag-1 activated kinase properties of c-Raf through binding to Ras, 14-3-3 and Bcl-2. Although cells were exposed to Cisplatin, forced expression of Bag-1L increased c-Raf levels in association with stable Bcl-2 expression profile and led to activation of anti-apoptotic machinery in HeLa cells. In similar way, we found that Cisplatin treatment for 24h downregulated c-Raf expression levels, but not alter the Ras expression level in HeLa wt cells. In contrary, forced Bag-1L expression prevented downregulation of c-Raf and promoted Ras expression levels after Cisplatin treatment. We first checked the expression levels of Hsps; Hsp90, Hsp70, Hsp60, Hsp40 and HSF1 in both cell lines. Expression profiles of Hsp70 and Hsp60 were not altered after time-dependent Cisplatin treatment in wt and Bag-1L+ HeLa cells. However, Hsp90 and Hsp40 expression were found

increased in Bag-1L+cells compared to wt HeLa cells. It was suggested that Bag1 formed a stoichiometric complex with Hsp70 and inhibited completely Hsp70-dependent *in vitro* protein refolding of an unfolded polypeptide (Nollen et al., 2000). Contrary to this finding, neuroprotective role of forced Bag-1 expression was found associated with the activation of Hsp70 to proceed cell survival (Liman et al., 2005). Similar to this finding, we concluded that although Cisplatin did not reduce Hsp70 expression level, Bag-1L stable transfection might increase chaperone activity of Hsp70 and prevented c-Raf downregulation after Cisplatin treatment. Moreover, it was suggested that Hsp70 could protect the cells from energy deprivation and/or ATP depletion resulted with apoptosis. Hsp70 could also chaperone altered protein products generated upon caspase activation. Thus, stable expression of Hsp might rescue cells in the later phase of the apoptosis signaling cascade and enhanced the Bag-1L mediated cytoprotectivity. Although direct role of Bag-1L on Hsp60 was not established fully, Cisplatin treatment did not alter Hsp60 expression in wt and Bag-1L+HeLa cells. Similar to previous reports, Hsp60 might enhance the tumor progression role of Bag-1 through stabilizating its regulatory co-chaperone role in malign cells. In addition Hsp60 was shown stress withstand molecule in microorganisms and here it might induce stress tolerance mechanism to maintain cell survival when the cells were exposed to Cisplatin (Conway de Macario and Macario 2003). Hsp40 regulates ATPase activity of Hsp70. Bag-1L forced expression in HeLa cells prevented Cisplatin-induced downregulation of Hsp40. This finding was also supported that stable transfection of Bag-1L promoted Hsp70 activity. However, upregulation of Hsp70 expression due to stress induction might result in the formation of Bag-1-Hsp70 complexes that could compete against Bag-1-Raf-1 complex formation, thus down-regulating Raf-1 kinase activity (Song et al., 2001). Therefore, protection of Raf-1 expression levels after Cisplatin treatment (Figure 4) was found critical to establish the supporting survival action of Bag-1L in HeLa cells. It was shown that chaperones Hsc70 and Hsp90 not only associated with mutant p53, but they also played important roles in regulating wt p53 activity during normal cell growth and stress conditions (King et al., 2001). They proposed that Hsp90 might support nuclear translocation of wt p53 and Bag-1 could dissociate wt p53-Hsp90 complex, led to proteolysis of p53. However, under stress stimuli, increased Hsp70 levels could result in the formation of Bag-1-Hsp70 complex in stress tolerated cells, which might in turn lead to the sequestration of Bag-1 and the subsequent import of wt p53-Hsp90 into the nucleus (King et al., 2001; Song et al., 2001; Zylicz et al., 2001).

In conclusion, Bag-1 is a critical mediator of drug resistance phenotype in HeLa cells exposed to Cisplatin through modulating its interacting molecules. For this reason the clarification of Bag-1 isoforms in drug-resistance mechanism could be determinative to understand the efficiency of therapy.

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