Published in final edited form as:

Exp Cell Res. 2012 October 1; 318(16): 1973–1986. doi:10.1016/j.yexcr.2012.06.011.

DOWNREGULATION OF SWI/SNF CHROMATIN REMODELING FACTOR SUBUNITS MODULATE CISPLATIN CYTOTOXICITY

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Abstract

Chromatin remodeling complex SWI/SNF plays important roles in many cellular processes including transcription, proliferation, differentiation and DNA repair. In this report, we investigated the role of SWI/SNF catalytic subunits Brg1 and Brm in the cellular response to cisplatin in lung cancer and head/neck cancer cells. Stable knockdown of Brg1 and Brm enhanced cellular sensitivity to cisplatin. Repair kinetics of cisplatin DNA adducts revealed that downregulation of Brg1 and Brm impeded the repair of both intrastrand adducts and interstrand crosslinks (ICLs). Cisplatin ICL-induced DNA double strand break repair was also decreased in Brg1 and Brm depleted cells. Altered checkpoint activation with enhanced apoptosis as well as impaired chromatin relaxation was observed in Brg1 and Brm deficient cells. Downregulation of Brg1 and Brm did not affect the recruitment of DNA damage recognition factor XPC to cisplatin DNA lesions, but affected ERCC1 recruitment, which is involved in the later stages of DNA repair. Based on these results, we propose that SWI/SNF chromatin remodeling complex modulates cisplatin cytotoxicity by facilitating efficient repair of the cisplatin DNA lesions.

Keywords

Brg1; Brm; chromatin remodeling; cisplatin

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cause of cancer deaths. The 5-year survival rate of HNSCC patients is less than 50% [1]. Lung cancer

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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accounts for the largest number of new diagnosis and deaths from cancer annually and non-small cell lung cancer (NSCLC) accounts for more than 75–80% of lung cancers [2]. The NSCLC patients have a 5-year survival rate of 15–17%. The prognosis for HNSCC and NSCLC is very poor and the management of these two types of cancer involves treatment with cisplatin alone or in combination with other chemotherapeutic drugs, radiation therapy and/or surgery [3,4]. Randomized clinical trials have confirmed that adjuvant chemotherapy with platinum-based drug combinations for NSCLC significantly increases patient survival [2]. Lack of biomarkers for early detection and risk assessment affects the survival rate of these patients.

SWI/SNF, an important member of chromatin remodeling family is a multisubunit complex that depends on the key catalytic subunits, Brahma (Brm) or Brahma related gene 1 (Brg1) for its remodeling function. Brg1 and Brm are frequently silenced singly or concomitantly thereby impairing and/or inactivating the SWI/SNF complex in cancer cells and tumor samples including lung and head/neck cancers [5–7]. SWI/SNF activity alters chromatin structure by ATP-dependent disruption of histone-DNA interaction by displacing the histones or sliding the histones along the DNA. SWI/SNF is required for the regulation of transcription, cell cycle control, differentiation, proliferation and other signaling pathways in mammalian cells [8]. SWI/SNF also plays a major role in the repair of DNA lesions directly by facilitating DNA accessibility on the chromatin or indirectly by facilitating the functions of DNA repair proteins such as p53, BRCA1, GADD45 and Fanconi Anemia proteins [9–14]. Studies by us and others have shown that concomitant loss of both Brg1 and Brm was observed in 15–20% of NSCLC and 10–20% of HNSCC, which correlated with poor prognosis [6,7,15]. This suggests that Brg1 and Brm could be used as prognostic markers [16] and accumulating evidence identifies these proteins as tumor suppressors [17].

Cells lacking Brg1 have been shown to be sensitive to UV radiation and cisplatin and sensitivity to UV radiation has been associated with inhibition of the nucleotide excision repair (NER) pathway which removes the UV- DNA lesion [10,18]. *In vitro* studies show that nucleosomes impede excision of UV and cisplatin DNA adducts, while addition of the remodeling complex decreases the inhibition of UV damage excision [19,20]. In this report, we evaluate the effects of Brg1 and Brm downregulation on cisplatin cytotoxicity in human NSCLC and HNSCC cells. Downregulation of Brg1 and Brm resulted in enhanced cellular sensitivity to cisplatin. Incomplete repair of cisplatin intrastrand adducts, interstrand crosslinks (ICLs) and ICL-induced DNA double strand breaks (DSBs) was observed in Brg1 and Brm depleted cells. Brg1 and Brm deficiency resulted in impaired chromatin relaxation, altered checkpoint activation as well as enhanced apoptosis. The recruitment of XPC to cisplatin DNA lesions was unaffected, whereas recruitment of ERCC1 was affected following stable knockdown of Brg1 and Brm in these cells. These data indicate that Brg1 and Brm modulate cisplatin cytotoxicity in cancer cells via chromatin remodeling and facilitate the recruitment of DNA repair factors following DNA damage recognition.

MATERIAL AND METHODS

Chemicals

Cisplatin was purchased from Sigma Aldrich. All other chemicals and reagents were from standard suppliers. Brg1 antibody was from Santa Cruz Biotechnology, Brm antibody was from Abcam, ERCC1 antibody (FL297) was from Santa Cruz Biotechnology, XPC antibody was from Novus Biologicals, γ H2AX antibody was from Millipore and α -tubulin antibody was from Sigma-Aldrich. ICR4, a monoclonal antibody against cisplatin intrastrand adduct (GG) was kindly provided by Michael J. Tilby, University of New Castle, UK.

Cell lines

The human head and neck squamous-carcinoma cell lines, UM-SCC-5PT and UM-SCC-10BPT (kindly provided by Dr. Thomas E. Carey, University of Michigan) [21], and human non-small cell lung cancer cell line H460 (kindly provided by Dr. James C. Willey, University of Toledo) were maintained in RPMI-1640 supplemented with 10% FBS plus antibiotics and grown at 37°C in a 5% CO₂ incubator.

Transfection with shRNAs

We used lentiviral vectors (pLKO.1) expressing specific shRNA to target Brg1 or Brm. shRNA knockdown reagents were obtained from Sigma-Aldrich (Mission RNAi). Cells were seeded in six-well plates (2.5×10^5 cells/well) and transduced with lentiviral shRNA expression vectors. After incubating the cells overnight at 37°C, the virus containing media was replaced with fresh media. Selection was carried out for a week with 2 µg/mL and 5 µg/mL puromycin for H460 and UM-SCC cells, respectively. Knockdown of the proteins was confirmed by western blot (Supplemental Information), and further experiments were carried out using the wildtype and knockdown (KD) cells.

MTS assay

The CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega) was used to assess the cytotoxicity of cisplatin. Cells were treated with increasing doses of cisplatin for 2 hr. After 72 hr, 20 µL of combined MTS/PMS solution [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and phenazine methosulfate] was added to each well and incubated for 2–4 hr at 37°C. The absorbance was measured at 490 nm in Spectramax M5 plate reader (Molecular Devices). We used blank medium without cells to correct background and subtracted the OD of blank from OD of treated as well as control cells. The percentage of cell survival was calculated from the average OD of treated cells/average OD of control cells x 100.

Cisplatin intrastrand adduct measurement

Repair of intrastrand adducts was assessed by ELISA as described with some modifications [22,23]. Cells were treated with cisplatin for 2 hr and genomic DNA was isolated at indicated time points using Qiagen DNeasy blood and tissue kit as per the manufacturer's protocol. Equal amounts of DNA was coated on 96 well ELISA plates and probed with ICR4 antibody. After incubation with HRP conjugated goat anti-rat antibody (Calbiochem), TMB (1 step ultra TMB-ELISA, Thermo Scientific) was added. The reaction was stopped with 2M sulfuric acid and absorbance was measured at 450 nm (Spectramax M5 plate reader, Molecular Devices). The % intrastrand adducts were calculated using OD 450 nm where the 0 hr time point was used as 100% intrastrand adducts in each cell line.

Cisplatin interstrand crosslink measurement

Repair of interstrand crosslinks was assessed by alkaline comet assay with some modifications [22,23]. Cells were treated with cisplatin for 2 hr and harvested at indicated time points. Cells were further treated with hydrogen peroxide for 15 min to induce DNA strand breaks. Cell suspensions (~10000 cells) were embedded on a microscopic slide, lysed and incubated in ice-cold alkaline solution for 20 min. Electrophoresis was carried out for 25 min at 28 V, 300 mA. Slides were neutralized and stained with SYBR green (Trevigen). The comets were scored using a Nikon epifluorescence microscope. At least fifty cells were analyzed per slide using Komet Assay Software 5.5F (Kinetic Imaging, Liverpool, UK). The data was expressed as the percentage of crosslinks that remained at that particular time point normalized to 100% at 0 hr.

Flow cytometry

Cell cycle analysis was performed by flow cytometry. Cells were synchronized with aphidicolin (3 μ M) and treated with cisplatin for 2 hr. At indicated time points, cells were pelleted and washed with PBS. Cells were fixed in ice-cold ethanol and stained with propidium iodide (PI/RNase staining buffer, BD Pharmingen). DNA content was analyzed on a FACSCalibur flow cytometer (BD Biosciences) with CellQuest software. The cell cycle profile was determined with Modfit LT software.

Apoptosis assay

Cisplatin induced apoptosis was determined using the Guava Nexin kit (Guava Technologies) according to the manufacturer's instructions. Briefly, cells were treated with cisplatin for 24 hr and washed with PBS. For each sample, 10^5 cells were collected and incubated with Guava Nexin reagent for 20 min in the dark to stain for Annexin V and 7-amino-actinomycin D (7-AAD). Apoptotic cells were analyzed with Guava PCA system using cytosoft, where early apoptotic cells are positive for Annexin V and late apoptotic cells are positive for both Annexin V and 7-AAD. The percentage of apoptosis was calculated from the early and late apoptotic cells in each sample.

Chromatin relaxation

Micrococcal nuclease (MNase) digestion assay was performed to assess chromatin relaxation as described [24]. Cells were treated with cisplatin for 2 hr and lysed in hypotonic lysis buffer (10mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 25% glycerol, 0.2% NP-40 and protease inhibitors) for 30 min. Nuclei were isolated by centrifugation and resuspended in 200 μ l MNase buffer (10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$) and digested with MNase for 5 min at room temperature. The reaction was stopped by stop solution (1% SDS, 20 mM EDTA). DNA was isolated by phenol/chloroform and separated on a 1% agarose gel.

Immunofluorescence

Colocalization of XPC and ERCC1 with cisplatin DNA adducts was assessed by immunofluorescence as described [25]. Cells were grown on coverslips and treated with cisplatin for 24 hr. Cells were fixed with 4% paraformaldehyde in 0.2% TritonX-100/PBS for 20 min on ice. After 3 washes in PBS, the DNA was denatured by 2N HCl for 20 min at 37°C. Cells were incubated in 20% fetal bovine serum in washing buffer (0.1% Triton X-100/PBS) for 1 hr at room temperature. Primary and secondary antibodies were prepared in 1% BSA in washing buffer. Antibodies against cisplatin GG intrastrand adduct, ERCC1 and XPC were incubated for 1 hr at room temperature. After 3 washes in wash buffer for 5 min, secondary antibodies conjugated to Alexafluor 488 and 568 were incubated for 1 hr at room temperature. Cells were washed and counterstained with DAPI for 5 min. Coverslips were mounted with DAKO mounting medium onto the slides. Images were captured by Nikon Eclipse T2000-U microscope and percent co-localization of XPC and ERCC1 with GG adduct was calculated.

Statistical analysis

Statistical analysis was performed using a paired t-test on R version 2.7.0. Statistical comparisons are made between wildtype and KD cells.

RESULTS

Stable knockdown of Brg1 and Brm

The expression of Brg1 and Brm was studied in lung cancer (H460) and head/neck cancer (5PT and 10BPT). Cells were stably transfected with Brg1 or Brm shRNA and stable clones/transfectants were selected using Puromycin. The expression levels of Brg1 and Brm were analyzed, using α -tubulin as a loading control. As seen in Supplemental Figure 1 (Supplemental Information), we achieved a significant decrease in Brg1 or Brm protein levels in shRNA transfected (KD) cells compared to untransfected (wildtype) controls.

Downregulation of Brg1 and Brm increases cisplatin sensitivity

To determine the potential role of SWI/SNF on cell viability in response to cisplatin, MTS assays were performed in wildtype, Brg1 KD and Brm KD cells (Figure 1). Downregulation of Brg1 and Brm significantly enhanced cisplatin sensitivity in both lung cancer and head/neck cancer cells. We observed ~ 3.5 and ~ 2.2 fold increase in cisplatin sensitivity followed by knockdown of Brg1 and Brm, respectively in H460 cells as shown in Figure 1A. In 5PT and 10BPT, down regulation of Brg1 results in ~ 3.5 fold change in IC₅₀ values (Figure 1B, C). SWI/SNF defective cells have been shown to be more sensitive to DNA damaging agents such as UV radiation, cisplatin, doxorubicin, etoposide and IR radiation [10,18,26,27]. Our results show that SWI/SNF is important for cell survival after cisplatin treatment and the expression levels of catalytic subunits of SWI/SNF complex could be an important determining factor in mediating cisplatin sensitivity.

Defective cisplatin intrastrand adduct and ICL repair in Brg1 and Brm deficient cells

Accumulating evidence supports a role for the SWI/SNF complex in the repair of DNA damage. SWI/SNF modulates cytotoxicity of DNA damaging agents by facilitating DNA repair [24,25,27–29]. Therefore, we asked if SWI/SNF can promote repair of cisplatin DNA lesions. Cisplatin exerts its cytotoxicity by reacting with DNA and forming monoadducts, intrastrand adducts and interstrand crosslinks (ICL) [30]. Therefore, we examined the repair kinetics of cisplatin intrastrand adducts and ICLs following Brg1 and Brm downregulation. First, we monitored the percent of intrastrand adducts repaired over time by an ELISA method, using a monoclonal antibody specific for the major cisplatin GG intrastrand adduct (Figure 2). Wildtype cells showed gradual repair of cisplatin GG adducts with increasing time (from 24 to 48 hr) in both lung cancer and head/neck cancer cells. In Brg1 and Brm KD cells, the rate of removal of these adducts was decreased significantly. Almost 50% of these adducts still remained/persisted at 48 hr in Brg1 and Brm deficient cells. We then monitored the repair kinetics of cisplatin ICLs at different time intervals by alkaline comet assay and plotted the data as the percentage of crosslinks remaining at a particular time point. Figure 3 shows the percentage of cisplatin ICLs with increasing time in wildtype and KD cells. Similar to intrastrand adducts, cisplatin ICLs were repaired gradually from 24 hr to 72 hr in wildtype cells with only 20% of ICLs remaining at 72 hr. However, more than 40% of ICLs remained at 72 hr in Brg1 and Brm depleted cells. These data show that loss of Brg1 and Brm results in inefficient repair of cisplatin intrastrand adducts as well as ICLs suggesting the involvement of SWI/SNF in stimulating the repair of cisplatin DNA lesions.

It has been shown that cisplatin ICL processing generates DNA double strand breaks (DSBs). Histone variant H2AX is phosphorylated (γ H2AX) and forms distinct nuclear foci at the sites of DSBs. The disappearance of γ H2AX correlates with the repair of ICL-induced DSBs [31]. Therefore, we monitored kinetics of γ H2AX foci repair in wildtype and knockdown cells to further support and corroborate the results of the comet assay. Supplemental Figure 2 shows the percentage of γ H2AX foci positive cells over a period of time after cisplatin treatment, which denotes the percentage of DSBs remaining at different

time intervals in lung cancer and head/neck cancer cells. In wildtype cells, γ H2AX foci peaked at 24 hr post-cisplatin treatment and were subsequently repaired at 48 and 72 hr. However, in Brg1 and Brm KD cells, repair of γ H2AX foci was significantly decreased after 24 hr. This shows that ICL-induced DSB repair is incomplete upon downregulation of Brg1 and Brm. This is consistent with previous studies indicating a role for SWI/SNF complex in DSB repair [29,32].

Perturbation of checkpoint activation in Brg1 and Brm deficient cells

Following treatment, cisplatin induces a DNA damage response resulting in cell cycle checkpoint activation [33]. To determine the effect of Brg1 and Brm deficiency on activation of cell cycle checkpoints, we analyzed the DNA content of wildtype and KD cells synchronized with aphidicolin. As shown in Figure 4, wildtype cells gradually progressed into G2/M phase of the cell cycle from 0 to 72 hr. At 48 and 72 hr, more than 40% of the cells accumulated in the G2/M phase. This is consistent with previous studies, where G2/M DNA damage checkpoint is activated after cisplatin treatment [34]. In wildtype cells, the G2/M checkpoint activation is sufficient in time to protect the cells from inducing an apoptotic response. This is reflected in the low level of apoptotic cells in wildtype after cisplatin treatment until 72 hr. In both Brg1 and Brm deficient cells, this G2/M arrest was not observed. Instead, a large proportion of the KD cells accumulated at the G1 phase with a concomitant increase in apoptosis. Several studies show aberrant cell cycle checkpoint activation in the absence of SWI/SNF complex [10,26,27]. Cell cycle perturbation caused by a G1 block or failure to activate the G2/M DNA damage cell cycle checkpoint could have resulted in enhanced apoptosis in the Brg1 and Brm KD cells.

To further confirm this, we performed Guava Nexin assays which utilize Annexin V-PE staining to detect apoptosis at the early stage and 7-ADD staining as an indicator of cell membrane structural integrity. Table 1 shows the percentage of apoptotic cells in cisplatin-treated wildtype and KD cells. Brg1 and Brm depletion significantly enhanced cisplatin-induced apoptosis compared to wildtype cells. This increased apoptosis could be due to the failure to induce G2/M checkpoint activation resulting in excessive DNA damage and cell death [26].

Impaired chromatin relaxation in Brg1 and Brm deficient cells

SWI/SNF is an ATP-dependent chromatin remodeling complex that alters chromatin structure in response to DNA damage. Efficient repair of DNA damage requires unfolding of the chromatin into a more relaxed state [35]. As seen in Figures 2 and 3, incomplete repair of cisplatin intrastrand adducts and ICLs was observed in Brg1 and Brm deficient cells. Therefore, we performed micrococcal nuclease (MNase) assays to evaluate the effect of SWI/SNF on chromatin decondensation as relaxed chromatin is more sensitive to nuclease digestion. Wildtype and KD cells were treated with cisplatin and nuclei were isolated for digestion with MNase followed by separation of DNA on agarose gels. Figure 5 represents the MNase sensitivity assay in cisplatin treated lung cancer and head/neck cancer cells. MNase digestion resulted in distinct banding patterns showing mono, di, tri and polynucleosomes compared to control cells (without MNase). Cisplatin treatment rendered chromatin more susceptible to MNase digestion and enhanced degradation of chromatin into oligonucleosomal DNA ladders in wildtype cells. However, both Brg1 and Brm KD cells treated with cisplatin were less sensitive to MNase showing impaired chromatin relaxation. This indicates that SWI/SNF is required for chromatin relaxation and deficiency of Brg1 and Brm results in impaired chromatin decondensation. Our results are consistent with previous reports, where Brg1 deficient cells are defective in UV-induced chromatin relaxation [24].

Recruitment of XPC and ERCC1 to cisplatin DNA adducts in Brg1 and Brm deficient cells

It is well known that lesion detection is important for efficient DNA damage repair [36]. The role of SWI/SNF complex on DNA damage recognition remains largely inconclusive [9,37]. Nucleotide excision repair (NER) is a versatile DNA repair pathway that removes bulky DNA lesions that distort the double helix structures such as cisplatin DNA adducts. NER is mediated by DNA lesion detection, localized DNA unwinding, endonuclease recruitment and incision which is then followed by repair synthesis [38]. XPC is a crucial DNA damage recognition protein that initiates global genome NER. Photo cross-linking studies show that XPC binds to cisplatin DNA adducts (1,2 GpG intrastrand) *in vitro* [39] and is required for cisplatin-DNA repair [40]. ERCC1 is a critical structure specific endonuclease that performs the incision of the damaged strand during the NER reaction and the absence of which severely affects the repair of cisplatin damaged DNA [22,23].

Therefore, we assessed the effect of Brg1 and Brm on the recruitment of XPC and ERCC1 to the cisplatin DNA damaged sites. We used a monoclonal antibody against the cisplatin 1,2 GG intrastrand adduct to detect co-localization with XPC and ERCC1 in cisplatin treated wildtype and KD cells. Figures 6 and 7 show immunofluorescent detection and percent co-localization of XPC and ERCC1 with GG adduct foci in lung cancer cells. Co-localization experiments in head/neck cancer cells treated with cisplatin were also conducted (Supplemental Figures 4 and 5). In wildtype cells, distinct co-localization of XPC as well as ERCC1 with GG adducts was observed as seen in the figure indicating recruitment of both early and later stage NER factors to cisplatin DNA lesions, respectively. Similar co-staining of XPC and GG adduct was observed in Brg1 and Brm KD cells as well. Recruitment of XPC to cisplatin GG intrastrand adducts was comparable in both wildtype and KD cells with ~ 90% co-localization (Figure 6 and Supplemental Figure 4). However, recruitment of ERCC1 to cisplatin GG adducts was significantly decreased in KD cells with less than 20% co-localization compared to wildtype cells (Figure 7 and Supplemental Figure 5). Contrasting results exist with studies showing SWI/SNF affects recruitment of XPC to UV damaged DNA sites [24,25,28]. Our results suggest that the SWI/SNF complex does not influence cisplatin DNA lesion detection and hence could function downstream of DNA damage recognition. This is an important observation since it addresses the long-standing debate of whether chromatin relaxation precedes lesion detection or vice versa [35,41]. The inefficient co-localization of ERCC1 to cisplatin DNA lesions in the absence of Brg1 and Brm indicate that chromatin remodeling affects later stage protein recruitment required for efficient DNA repair. The deficient ERCC1 recruitment is consistent with the incomplete repair of cisplatin DNA lesions observed in the KD cells (Figures 2 and 3).

DISCUSSION

The chromatin remodeling complex SWI/SNF contains either of the two catalytic ATPase subunits, Brm (Brahma) or Brg1 (Brm-related gene) together with 9–12 Brg1/Brm associated factors (BAF) [8,42–44]. The components of the SWI/SNF complex interact with various proteins from different DNA repair pathways and modulate the repair of DNA adducts induced by DNA damaging agents [24,25]. Chromatin is the main target of DNA damaging agents and the chromatin structure affects the repair processes. DNA is wrapped around histones and the packaged nucleosomes can impede the repair of the DNA lesions [37]. It has been shown that the excision of UV and cisplatin adducts from nucleosomal DNA was significantly less than the excision from free or naked DNA [19,36]. The addition of chromatin remodeling complex decreased the extent of this inhibition *in vitro*, providing evidence for the importance of chromatin remodeling in repairing DNA lesions [41]. The SWI/SNF complex directly modifies the nucleosome structure by disrupting the histone-DNA interactions in an ATP-dependent manner. This alteration facilitates the accessibility of DNA repair enzymes to the damaged DNA sites [9]. In this study, we demonstrate that

Brg1 and Brm, the subunits of the SWI/SNF complex are important for chromatin relaxation followed by cisplatin treatment as loss of these proteins exhibits impaired chromatin decondensation as shown by MNase sensitivity assays (Figure 5). This is expected as compact chromatin structure unwinds to a relaxed state in response to DNA damage [24,35,45]. However, Brg1 and Brm do not influence cisplatin DNA damage recognition as evidenced by the comparable recruitment of XPC to cisplatin DNA lesions in both wildtype and KD cells (Figure 6 and Supplemental Figure 4). Studies show that Brg1 and tumor suppressor protein p33ING2 are essential for the recruitment of DNA damage recognition proteins XPC and XPA to UV damaged DNA sites [25,45]. However, other studies demonstrate that components of SWI/SNF complex do not affect co-localization of XPC to UV lesions [24,28]. We show that cisplatin DNA lesion detection is independent of chromatin relaxation in support of DNA damage recognition preceding chromatin relaxation.

Since cellular responses to cisplatin also include checkpoint activation, we determined whether SWI/SNF is required for activation of cell cycle arrest following cisplatin treatment. Components of the SWI/SNF complex have been implicated in the control of cell growth and proliferation and inactivation of the complex results in defective cell cycle checkpoint activation [10,26,27]. When cells encounter cisplatin DNA damage, the cell cycle checkpoint is activated by arresting the cells at the G2/M phase to allow DNA repair, the failure of which results in apoptotic cell death. Loss of Brg1 has been shown to inhibit retinoblastoma mediated cell cycle arrest in cancer cells [46] and result in genomic instability in mice [47]. In the current study, deficiency of Brg1 and Brm resulted in aberrant cell cycle checkpoint activation accompanied by enhanced apoptosis (Figure 4, Table 1). The increased accumulation of cisplatin damaged cells at the G1 phase likely results in apoptotic signaling due to the inability of the cells to repair the cisplatin DNA lesions. Therefore, enhanced sensitivity to cisplatin in Brg1 and Brm depleted cells (Figure 1) could be due to inactivation of cell cycle checkpoint as well as incomplete DNA repair.

Several studies have shown that mammalian SWI/SNF complex stimulates the repair of UV radiation, doxorubicin, etoposide and IR induced DNA damage [10,24,26,27]. The absence of Brg1 was shown to inhibit NER of UV adducts and increase apoptosis and cell sensitivity, which was attenuated by ectopic expression of Brg1 [10]. Although chromatin structure has been shown to inhibit cisplatin DNA adduct repair *in vitro* [19], this has not been studied in cells by targeting the chromatin remodeling factors. In this report, we show that disruption of Brg1 and Brm results in inefficient repair of cisplatin intrastrand adducts, ICLs and ICL-induced DSBs (Figures 2, 3, and Supplemental Figure 2). Intrastrand adducts are primarily repaired by the NER pathway [48], while ICL repair, in addition to NER, requires homologous recombination (HR) [49]. In SWI/SNF deficient cells, cisplatin ICLs were more refractory to repair compared to intrastrand adducts. The majority of cisplatin ICL repair is replication-dependent and SWI/SNF deficient cells fail to leave the G1 phase of the cell cycle indicating replication arrest (Figure 4), likely resulting in incomplete repair of cisplatin ICLs.

We and others have shown that downregulation of NER factors XPF and ERCC1 results in cisplatin hypersensitivity, accompanied by extreme defects in repair of both types of cisplatin lesions [22,23]. Downregulation of Brg1 and Brm affected the recruitment of ERCC1 to the cisplatin damaged sites in this study (Figure 7 and Supplemental Figure 5). This indicates that the SWI/SNF complex is essential for NER events downstream of DNA damage recognition. The extent of inhibition of cisplatin adducts in the present study is not as pronounced or severe in comparison to NER deficiency [22]. This suggests that unlike NER proteins, the SWI/SNF remodeling factors are not directly involved in the repair of cisplatin DNA damage. These factors alter the chromatin structure and facilitate the repair

by allowing accessibility of the repair factors to the sites of DNA damage [37,50]. In addition, cisplatin DNA lesions formed in the linker regions of chromatin likely do not require the same extent of relaxation for the repair process. Since NER is the major cisplatin DNA repair pathway, it is reasonable to postulate that SWI/SNF facilitates the NER pathway in repairing cisplatin DNA lesions. Since, SWI/SNF complex is involved in transcriptional regulation, we wanted to check if the absence of Brg1 and Brm affected transcription of NER factors. We found that transcriptional regulation of early and late stage NER factors is not affected by downregulation of Brg1 and Brm (Supplemental Figure 6) indicating that NER protein availability was intact in Brg1 and Brm deficient cells. This clearly demonstrates that the incomplete repair of cisplatin DNA lesions is not due to transcriptional regulation of NER factors by the SWI/SNF complex.

Based on our results, we propose a model that the SWI/SNF chromatin remodeling complex modulates cisplatin cytotoxicity (Figure 8). Following cisplatin treatment, DNA damage recognition factors detect cisplatin DNA lesions which could facilitate chromatin relaxation by the SWI/SNF complex. This chromatin relaxation and subsequent checkpoint activation modulates accessibility and recruitment of downstream DNA repair proteins to the damaged DNA sites. Successful completion of repair processing results in tolerance to cisplatin treatment. Targeting the SWI/SNF chromatin remodeling factor subunits Brg1 and Brm leads to impaired chromatin decondensation, altered checkpoint activation and incomplete repair of the cisplatin-DNA adducts which results in enhanced cisplatin sensitivity.

CONCLUSION

Chromatin remodeling complex SWI/SNF plays major roles in important cellular functions and functional interference to this complex can result in mutations and chromosomal aberrations that can increase the risk of developing cancer. The core subunits of the complex, Brg1 and Brm are identified as tumor suppressors and their loss has been shown to induce tumorigenesis. In addition, deregulation of these proteins has been reported in cancer patients. Regulation of DNA repair by the chromatin remodeling complex as shown in the present study can be utilized for efficient chemotherapeutic strategies. Cisplatin has been successfully used alone or in combination with other chemotherapeutic drugs for the treatment of various malignancies. It is established that DNA repair of cisplatin lesions can be detrimental to the cytotoxic efficacy of the drug. We show that loss of Brg1 and Brm can impede the repair of cisplatin DNA lesions and initiate apoptosis in head/neck and lung cancer cells. Therefore, Brg1 and Brm could be potential targets in tumors over expressing these factors to improve treatment. In addition, cisplatin chemotherapy could be effective in Brg1 and Brm negative tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank members of the Patrick lab for critical reading of the manuscript. We also thank Dr. Ivana de la Serna for helpful suggestions and providing antibodies and primers. This study was supported by grants from the American Cancer Society (RSG-06-163-01) and the National Institutes of Health (GM088249) both awarded to SMP.

Abbreviations

NSCLC non-small cell lung cancer

HNSCC	Head and neck squamous cell carcinoma
NER	nucleotide excision repair
HR	homologous recombination
ERCC1	excision repair cross-complementation group 1
XPC	xeroderma pigmentation group C
ICL	interstrand crosslinks
ELISA	enzyme linked immuno-absorbent assay
DAPI	4'-6-diamidino-2-phenylindole dihydrochloride

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Highlights

Stable knockdown of Brg1 and Brm enhances cellular sensitivity to cisplatin.

Downregulation of Brg1 and Brm impedes the repair of cisplatin intrastrand adducts and interstrand crosslinks.

Brg1 and Brm deficiency results in impaired chromatin relaxation, altered checkpoint activation as well as enhanced apoptosis.

Downregulation of Brg1 and Brm affects recruitment of ERCC1, but not XPC to cisplatin DNA lesions.

Figure 1. Cisplatin sensitivity

Wildtype, Brg1 KD and Brm KD cells were treated with increasing concentrations of cisplatin for 2 hr and cell viability was determined by MTS assay (A, H460; B, UM-SCC-5PT; C, UM-SCC-10BPT). Cisplatin IC_{50} values are calculated in wildtype and KD cells. Values are represented as mean $\pm$ SD of three independent experiments.

Figure 2. Repair of cisplatin intrastrand adducts

Wildtype, Brg1 KD and Brm KD cells were treated with cisplatin 2 hr and genomic DNA was isolated at different time intervals (0, 24, and 48 h). ELISA was performed as described using a cisplatin GG intrastrand adduct antibody and the percentage of intrastrand adducts remaining was calculated (A, H460; B, UM-SCC-5PT; C, UM-SCC-10BPT). The results are represented as mean $\pm$ S.E.M of three independent experiments. * - $P < 0.05$, ** - $P < 0.01$.

Figure 3. Repair of cisplatin ICLs

Wildtype, Brg1 KD and Brm KD cells were treated with cisplatin for 2 hr and alkaline comet assay was performed as described at different time intervals (0, 24, 48 and 72 hr). The percentage of interstrand crosslinks remaining was calculated using olive tail moments (A, H460; B, UM-SCC-5PT; C, UM-SCC-10BPT). Results are represented as mean $\pm$ S.E.M of two independent experiments. * - $P < 0.1$, ** - $P < 0.05$, *** - $P < 0.01$.

Figure 4. Cell cycle analysis

Wildtype, Brg1 KD and Brm KD cells were synchronized with aphidicolin, treated with cisplatin for 2 hr and incubated at indicated time points. Cells were fixed and stained with propidium iodide. DNA content was determined by flow cytometry. The percentage cell population in different phases of cell cycle (sub-G1, G1, S, and G2/M) was quantified using Modfit LT software. (A, H460; B, UM-SCC-5PT; C, UM-SCC-10BPT).

Figure 5. Chromatin relaxation

Wildtype, Brg1 KD and Brm KD cells were treated with cisplatin for 2 hr and MNase sensitivity assay was performed to determine the extent of chromatin relaxation. (A, H460; B, UM-SCC-5PT; C, UM-SCC-10BPT). DNA ladder of 1kb plus was used as molecular weight marker (M).

Figure 6. Recruitment of XPC to cisplatin DNA lesions

A, Co-localization of XPC and cisplatin GG intrastrand adducts was detected by immunofluorescence. Wildtype, Brg1 KD and Brm KD cells were grown on glass coverslips and treated with cisplatin for 24 hr. Cells were fixed and immunostained for cisplatin GG adduct (green) and XPC (red), and co-staining is shown as merged. DAPI stained nucleus is shown in blue. B, Quantification of XPC-GG adduct co-localization. For each experiment, 100 cells were counted and the results are represented as the mean $\pm$ SD of 3 independent experiments. NS – non significant.

Figure 7. Recruitment of ERCC1 to cisplatin DNA lesions

A, Co-localization of ERCC1 and cisplatin GG intrastrand adducts was detected by immunofluorescence. Wildtype, Brg1 KD and Brm KD cells were grown on glass coverslips and treated with cisplatin for 24 hr. Cells were fixed and immunostained for cisplatin GG adduct (green) and ERCC1 (red), and co-staining is shown as merged. DAPI stained nucleus is shown in blue. B, Quantification of ERCC1-GG adduct co-localization. For each experiment, 100 cells were counted and the results are represented as the mean $\pm$ SD of 3 independent experiments. *** - $P < 0.001$.

Figure 8. Downregulation of SWI/SNF chromatin remodeling factor subunits modulate cisplatin sensitivity

Following cisplatin treatment, DNA damage recognition factors detect cisplatin DNA lesions which could facilitate chromatin relaxation by SWI/SNF complex. This could trigger cellular checkpoint activation and accessibility of damaged chromatin to the repair proteins involved in NER and HR pathways. Successful completion of repair processing results in tolerance to cisplatin treatment. Targeting SWI/SNF chromatin remodeling factors subunits Brg1 and Brm leads to impaired chromatin decondensation, altered checkpoint activation and incomplete repair of the cisplatin-DNA adducts which results in enhanced cisplatin sensitivity.

Cisplatin-induced apoptosis

Cells were treated with cisplatin for 24 hr and stained with Annexin V and 7-ADD. The percentage of apoptotic cells was calculated from the percentage of early and late apoptotic cells. Results are expressed as mean $\pm$ SD from 2 independent experiments.

Table 1

% of apoptotic cells		UM-SCC-5PT		UM-SCC-10BPT		H460	
wildtype	Brg1 KD	wildtype	Brg1 KD	wildtype	Brg1 KD	wildtype	Brg1 KD
12.73 $\pm$ 2.5	22.45 $\pm$ 1.4 *	12.13 $\pm$ 2.2	23.78 $\pm$ 1.2 *	13.15 $\pm$ 0.6	22.7 $\pm$ 1.8 *	21.8 $\pm$ 1.8 *	

* - P < 0.01